Preselective gene therapy for Fabry disease

Gangjian Qin*, Toshihiro Takenaka†, Kimberly Telsch†, Leslie Kelley*, Tazuko Howard‡, Thierry Levade§, Robert Deans¶, Bruce H. Howard‡, Harry L. Malech‖, Roscoe O. Brady†, and Jeffrey A. Medin*,,††**

*Department of Medicine, University of Illinois, Chicago, IL 60607; †Developmental and Metabolic Neurology Branch, National Institute of Neurological Disorders and Stroke, ‡Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, and ‖Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; §Laboratoire de Biochimie, Institut Louis Bugnard, Toulouse 31403, France; ¶Osiris Therapeutics, Baltimore, MD 21231; and **Department of Biochemistry and Molecular Biology, University of Illinois, Chicago, IL 60612

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Fabry disease is a lipid storage disorder resulting from mutations in the gene encoding the enzyme α-galactosidase A (α-gal A; EC 3.2.1.22). We previously have demonstrated long-term α-gal A enzyme correction and lipid reduction mediated by therapeutic *ex vivo* transduction and transplantation of hematopoietic cells in a mouse model of Fabry disease. We now report marked improvement in the efficiency of this gene-therapy approach. For this study we used a novel bicistronic retroviral vector that engineers expression of both the therapeutic α-gal A gene and the human IL-2Rα chain (huCD25) gene as a selectable marker. Coexpression of huCD25 allowed selective immunoenrichment (preselection) of a variety of transduced human and murine cells, resulting in enhanced intracellular and secreted α-gal A enzyme activities. Of particular significance for clinical applicability, mobilized CD34+ peripheral blood hematopoietic stem/progenitor cells from Fabry patients have low-background huCD25 expression and could be enriched effectively after *ex vivo* transduction, resulting in increased α-gal A activity. We evaluated effects of preselection in the mouse model of Fabry disease. Preselection of transduced Fabry mouse bone marrow cells elevated the level of multilineage gene-corrected hematopoietic cells in the circulation of transplanted animals and improved *in vivo* enzymatic activity levels in plasma and organs for more than 6 months after both primary and secondary transplantation. These studies demonstrate the potential of using a huCD25-based preselection strategy to enhance the clinical utility of *ex vivo* hematopoietic stem/progenitor cell gene therapy of Fabry disease and other disorders.

Fabry disease is an attractive target for somatic gene therapy. This is because metabolic cooperativity (or cross-correction) occurs whereby uncorrected "bystander" cells take up and use recombinant α-galactosidase A (α-gal A) secreted by engineered cells (1–3). In this way, small numbers of transduced cells can effect correction of a larger number of nontransduced cells *in vivo*. Functional uptake of α-gal A also provides the biological basis of enzyme-replacement therapy for Fabry disease (4). Tissues also may need only a fairly low percentage of normal α-gal A activity levels to reverse lipid storage (5). Furthermore, a murine model exists for the disorder (6) that allows *in vivo* testing of novel gene therapy approaches.

Our current approach to gene therapy for Fabry disease has been to target hematopoietic stem/progenitor cells for genetic manipulation to effect long-term α-gal A correction and systemic metabolic cooperativity. In related studies, we have demonstrated functional correction of the enzymatic defect in Fabry patient hematopoietic cells (7) and that specific secretion of α-gal A from transduced bone marrow mononuclear cells (BMMC) occurs *in vivo* (8). We also recently have demonstrated enzymatic correction and lipid reduction in clinically relevant organs of the Fabry mouse model after transplantation of BMMC transduced with a retroviral vector that engineers expression of human α-gal A (9).

The clinical applicability of the achievements noted above is limited in part by the relatively low transduction efficiency of human hematopoietic stem/progenitor cells by current regulatory-approved retroviruses. Although there are some inherited disorders such as severe combined immune deficiency in which correction of the genetic defect provides an *in vivo* growth advantage to some hematopoietic cell lineages (10, 11), for many disorders, including Fabry disease, there is not likely to be any growth advantage for therapeutically modified cells. Although another approach would be to reduce endogenous uncorrected hematopoietic cells by aggressive marrow conditioning before gene therapy, severely ill Fabry patients might not tolerate such treatments.

A number of effectors that allow selective enrichment of transduced cells have been engineered into retroviral vectors (12). Enrichment basically falls into two formats. One approach is to introduce genes such as *MDR*1 [multiple drug resistance 1 gene (13)] or mutated dihydrofolate reductase (14), which confer a survival advantage through resistance to selective drugs. However, a particular drug resistance gene may not confer a selective advantage sufficient to achieve a clinically relevant effect, especially where relatively quiescent cells such as hematopoietic stem cells are the target. This drawback may be the case either because there is an insufficient difference in resistance between transduced and nontransduced cells or because *in vivo* selection is not possible at tolerable drug doses in nonablated hosts (15). Additionally, some of the most effective drug-selectable genes are of heterologous origin [such as neomycin phosphotransferase (neo^R)] that can lead to host immune responses against transduced cells (16). There may be other types of unexpected toxicities as well. Overexpression of *MDR*1, for example, in mouse hematopoietic stem cells has been reported to cause myeloproliferative disorders in transplanted mice (17).

An alternative enrichment format is to engineer the expression of factors that allow preselection of transduced cells by flow cytometry or immunoaffinity means. Toward application in lipid storage disorders, Lorincz *et al.* (18) have derived a fluorescent enzymatic substrate and sorted transduced cells with high glucocerebrosidase (GC) activity. A number of groups also have used enhanced green fluorescent protein (enGFP) for this purpose. In a pioneering study in a murine model of protoporphyria, Pawliuk *et al.* (19) used a bicistronic vector containing a therapeutic gene and enGFP as a selectable marker. They sorted *ex vivo* transduced BMMC by flow cytometry and successfully repopulated recipient mice, leading to a cure of the disease phenotype. However, technical obstacles exist that may limit the clinical utility of this approach. Transduced cells must be sorted by flow cytometry, which currently is time-intensive and requires substantial manipulations of cells. Further, it has been shown that enGFP, a heterol-

Abbreviations: α-gal A, α-galactosidase A; GC, glucocerebrosidase; BMMC(s), bone marrow mononuclear cells; PBMC(s), peripheral blood mononuclear cells; huCD25, human IL-2Rα chain; enG(Y)FP, enhanced green (yellow) fluorescence protein; MSCs, mesenchymal stem cells; PB, peripheral blood; wt, wild type; NT, nontransduced; T/NS, transduced nonsorted; T/S, transduced sorted; IRES, internal ribosome entry site.

††To whom reprint requests should be addressed at: Section of Hematology/Oncology, University of Illinois, MBRB Room 3150 (M/C 734), 900 South Ashland Avenue, Chicago, IL 60607. E-mail: jmedin@uic.edu.

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ogous protein from jellyfish, elicits immune responses in mice (20) and likely will do so in other mammals including humans.

We sought to develop a selection strategy that may have more immediate clinical applicability. The coexpression of a therapeutic gene and a cell surface marker allows both flow cytometric and immunoaffinity bulk sorting of transduced cells. Clinical-scale immunoaffinity enrichment methods are well developed and currently are used in clinical transplantation protocols. An expressed cell surface marker also facilitates the systemic tracking of transduced cells. One example of this approach that has been well described uses the human CD24 or the equivalent murine heat stable antigen (HSA) markers (21–23). However, these markers may not be completely innocuous, as well, because HSA and CD24 are involved in the costimulation of T cells and in cell adhesion processes (24, 25). Other investigators have used the truncated human nerve growth factor receptor as a selectable marker (26), but this uses a surface molecule native to human cells but not normally expressed on hematopoietic cells.

We report here a preselection approach using a bicistronic retroviral vector that engineers coexpression of the therapeutic α-gal A gene along with the human IL-2Rα chain (huCD25) gene. We demonstrate successful immunoenrichment with associated enhancement of α-gal A activity after transduction of a variety of cells *in vitro*. These include Fabry patient fibroblasts and mobilized peripheral blood (PB) $CD34^+$ cells along with normal mesenchymal stem cells (MSCs). We tested the effects of this procedure *in vivo* in primary and secondary transplanted Fabry mice. Multilineage marking of PB cells in recipient animals was observed. Preselection of transduced BMMC led to higher percentages of functionally corrected cells that conferred a sustained increase of α-gal A activity in plasma and organs.

Materials and Methods

Bicistronic Therapeutic/Selective Vector Construct and Virus-Producing Cell Lines. Vector pMFG/α-gal A/IRES/huCD25 was derived from MFG (from R. Mulligan, Harvard Medical School). It was modified by standard molecular biology techniques to contain the full-length human α-gal A cDNA, the encephalomyocarditis virus internal ribosome entry site (IRES), and the huCD25 cDNA in tandem. It was fully sequenced to ensure fidelity. pMFG/α-gal A/IRES/huCD25 and plasmid pGT-N28 (New England Biolabs), which contain the neo^R gene, were cotransfected into packaging cell lines GP-E86 (27) and PG13 (28) by using a calcium phosphate method (7). Transfected PG13 and E86 cells were selected in G418 (Life Technologies, Gaithersburg, MD) and sorted by fluorescence-activated cell sorting (FACS Vantage; Becton Dickinson) for huCD25 expression. The brightest 5% of cells were diluted to isolate single cell clones. We isolated $\approx$200 individual clones for each packaging cell line. A secondary screen based on supernatant α-gal A activity then was performed and $\approx$40 clones were selected. These clones were titrated on NIH 3T3 (for E86) and HeLa (for PG13) cells. Two clones, E86/MFG/α-gal A/IRES/huCD25 clone 21 and PG13/MFG/α-gal A/IRES/huCD25 clone 50, were selected for use. Marker rescue assays (3), for the presence of replication-competent virus, were performed and found to be negative. We also used pUMFG/enYFP (29), which has the same vector backbone with a fluorescent reporter gene. This vector was packaged in PG13 and E86 analogously.

Isolation, Culturing, and Transduction of Human Cells. Granulocyte colony-stimulating factor-mobilized PB $CD34^+$ cells from a patient with Fabry disease were obtained by apheresis under an approved clinical protocol after obtaining informed consent. The peripheral blood mononuclear cells (PBMCs) were enriched for $CD34^+$ cells by positive immunoselection with the Isolex apparatus (Nexell Therapeutics, Irvine, CA) to $\approx$96% purity. For transduction, the cells were exposed to virus-containing supernatant five times in 4 days starting from day 0 in precoated fibronectin (2–5 $\mu g/cm^2$; Boehringer Mannheim) flasks in the presence of murine stem cell factor (50 ng/ml; R & D Systems), human IL-6 (10 ng/ml; R & D Systems), and protamine sulfate (Sigma). The cells then were maintained 2 additional days in culture before fluorescence-activated cell sorter analysis, enrichment, or colony assays. This procedure allowed expression from the transgene and not as a residual from fusion of the recombinant virion to the cell membrane (data not shown). Normal PBMCs and MSCs were obtained from PB and bone marrow (BM) donors, respectively. Simian virus 40-immortalized Fabry patient skin fibroblasts were generated as in ref. 30. ECV-304, HEL, HeLa, KG1, Jurkat, and HH cells were obtained from American Type Culture Collection (Manassas, VA).

Flow Cytometric Analyses. Cells were collected and washed in D-PBS (BioWhittaker). Indiscriminate antibody binding was blocked with 20% normal rabbit serum (Sigma) in D-PBS (blocking solution) for 20 min at 4°C, at a dilution of 50 $\mu l/10^5$–10^6 cells. Primary antibody then was added directly and incubated with the cells for 30 min at 4°C. The cells were washed with staining buffer [5 mM EDTA/1% BSA (Sigma) in PBS] and collected by centrifugation at 500 $\times$ *g* for 5 min at 4°C. The cells were resuspended in 50 μl of blocking solution with appropriate fluorescent-conjugated secondary antibody and incubated for 15 min at 4°C. The cells then were washed two times with staining buffer, collected, and resuspended in 0.2–1 ml of staining buffer for flow cytometric analysis. Propidium iodide (Sigma) at a 1 $\mu g/ml$ final concentration was added to exclude dead cells. Flow cytometric analyses were performed using a FACScalibur (Becton Dickinson).

Immunoaffinity Sorting for pMFG/α-Gal A/huCD25-Transduced Cells. For bulk immunoaffinity enrichment of transduced cells, we used magnetic microbead sorting and the manufacturer's protocol (MACS; Miltenyi Biotec, Auburn, CA). Briefly, cells were collected and blocking was performed as above. The primary antibody (anti-huCD25, clone 2A3; Becton Dickinson) was added at a concentration of 10 $\mu l/10^6$ cells. The cells were washed with PBS and collected by centrifugation. The cell pellet was resuspended in sorting buffer (PBS with 2 mM EDTA and 0.5% BSA) at a dilution of 80 $\mu l/10^7$ cells. Next, goat anti-mouse IgG microbeads (Miltenyi Biotec) were added at a concentration of 20 $\mu l/10^7$ cells and incubated for 20 min at 4°C. The cells were collected, washed, and applied to a magnetic cell separator (Miltenyi Biotec). Unbound cells were washed away. Positively selected cells were eluted into sorting buffer and counted, as were pass-through cells for an estimate of yield. To determine purity, a fraction of the sorted or flow-through cells was stained (as above) with another of two antibodies: huCD25-PC5 (B1.49.9) (Immunotech, Miami, FL) or Clone IL-2R1 (1HT44H3) (Coulter), which recognize different epitopes of huCD25. These discriminating antibodies were established by interference assays (data not shown).

Cell Proliferation Assay. To begin to gauge the effect of huCD25 overexpression on human lymphocyte proliferation, we performed [H^3]thymidine incorporation assays on transduced and control Jurkat cells (CD25-positive), HH cells (CD25-deficient), and HEL cells (nonlymphoid) in the presence of various concentrations of human IL-2 (kindly supplied by D. Peace, University of Illinois at Chicago). Cells (10^3) of either transduced and sorted or nontransduced origin were plated (in triplicate) in 100 μl of medium in each well of 96-well plates with serial concentrations of IL-2. On day 3, 1 μCi [H^3]thymidine (Amersham Pharmacia) was added to each well. After a 4-h incubation, the cells were washed three times, transferred onto a membrane (EasyTab-C; Packard), and measured for cpm (TopCount NTX; Packard).

α-Gal A and GC Enzyme Assays. α-Gal A activity was assayed fluorimetrically (9). Samples were added to a microtiter plate reader (Dynex, Chantilly, VA) and read for fluorescence against

nine independent dilutions of a 4-methylumbelliferone standard (Sigma). Media and plasma were collected, filtered, and assayed directly. Frozen mouse tissue samples were homogenized before sonication. As an additional control, GC activity was measured in parallel (23).

Isolation, Transduction, Enrichment, and Transplantation of Fabry Mouse BMMC. A colony of Fabry mice (6) was maintained under an approved protocol at the University of Illinois at Chicago. The genetic fidelity of offspring animals was verified by α-gal A assay on plasma collected from tail veins and by PCR-based genomic DNA assays on tail cuttings (9). Age- and parent strain (C57BL/6)-matched wild-type control mice were from the Jackson Laboratory. BMMC were obtained from the femurs and tibiae of 6- to 10-week-old male Fabry mice and transduced once a day for 5 days in DMEM medium (BioWhittaker) supplemented with 20% FCS (GIBCO/BRL) in the presence of recombinant murine stem cell factor (50 ng/ml; R & D Systems), murine IL-6 (10 ng/ml, R & D Systems), and protamine sulfate (6 μg/ml; Sigma) in flasks precoated with fibronectin (Boehringer Mannheim). After the last infection, cells were maintained in culture for 2 days. The cells then were subjected to flow cytometric analysis and immunoaffinity enrichment (as above). After sorting, the enriched cells were injected immediately via tail veins into lethally irradiated (1,100 rad) Fabry mice.

Colony-Forming Assays for Fabry Patient CD34$^+$ Cells and Murine BMMC. Sorted, nonsorted, and control transduced Fabry patient CD34$^+$ cells and Fabry mouse BMMC were plated at concentrations from 10^2 to 10^5 cells/ml in supplemented methylcellulose. For human CD34$^+$ cells, we used MethoCult GF H4435 (StemCell Technologies, Vancouver). For mouse BMMCs, we used MethoCult GF H3434 (StemCell Technologies). The numbers and types of colonies were counted at day 12 (murine) or day 16 (human) for each group. Flow cytometric analyses using huCD25 and lineage antibodies (PharMingen) were done on pooled and isolated, single colonies.

Results

Retroviral Vector pMFG/α-Gal A/IRES/huCD25. To implement preselective approaches for gene therapy for Fabry disease, we developed a novel recombinant retroviral vector (pMFG/α-gal A/IRES/huCD25) that allows enrichment of therapeutically transduced cells. We chose to use the MFG backbone because our earlier work demonstrated high expression with this construct (9). This new vector produces bicistronic mRNA with the α-gal A cDNA upstream of huCD25. In this way, cells selected on the basis of huCD25 expression should be obligate for expression of α-gal A. The use of an IRES sequence overcomes differential expression of two genes that can occur with dual internal promoters because of promoter interference (31). Use of huCD25 also facilitated virus-producer cell-line selection, screening, cloning, and titration analyses. We isolated an E86 clone for infection of murine cells and a PG13 clone for infection of human cells. The former had a titer of $\approx 5 \times 10^6$ productive infection units/ml units on NIH 3T3 cells, and the latter had a titer of $\approx 2 \times 10^6$ on HeLa cells.

Expression of huCD25 on Multiple Cell Types. First, we characterized anti-huCD25 cross-reactivity and surface staining on a variety of cells. Mouse cell lines, mouse PBMCs, and mouse BMMC showed no background staining to huCD25. Primary and simian virus 40-immortalized Fabry patient skin fibroblasts, the human endothelial cell line ECV-304, human myeloid HEL cell line, normal human primary MSCs, and human HeLa cells showed no background staining as well. The human myeloid cell line KG1 was strongly positive for huCD25. An Epstein–Barr virus-immortalized Fabry patient lymphoblastoid cell line (3) showed minimal staining. Human PBMCs, gated for lymphocytes, showed moderate staining of huCD25 (11–27%, $n = 3$) as expected, mainly arising from the CD4$^+$ population, whereas CD8$^+$ cells were minimally positive ($<2\%$, $n = 3$, data not shown). Granulocyte colony-stimulating factor-mobilized Fabry patient PB CD34$^+$ cells showed minimal huCD25 staining, accounting for $<0.5\%$ of the total CD34$^+$ cells. Control retroviral transduction itself was not found to substantially up-regulate huCD25 expression on Fabry patient CD34$^+$ cells (see Fig. 2), human fibroblasts, HEL, ECV-304 cells, or normal MSCs.

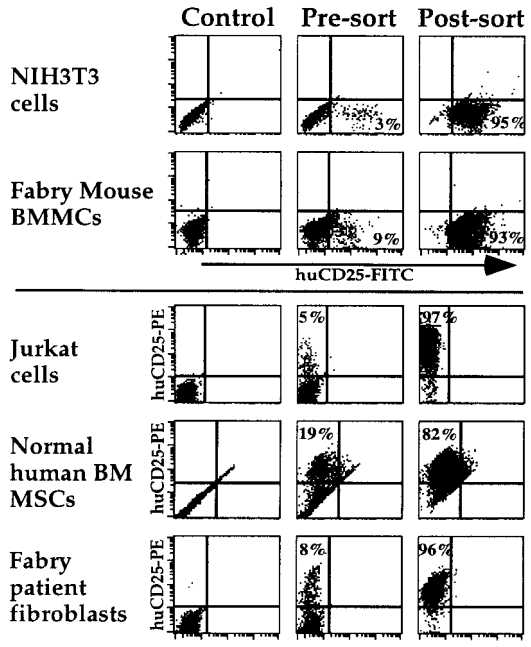

Fig. 1. Flow cytometry analyses after immunoaffinity enrichment of transduced cells. In some panels, FITC-conjugated anti-huCD25 antibody was used (FL1 channel, *x* axis); in others, phycoerythrin-conjugated antibody was used (FL2 channel, *y* axis).

Immunoaffinity Enrichment of Transduced Cells. The use of huCD25 as a cell surface marker enables rapid, efficient, and noninvasive selection of positively transduced cells to a high degree of purity. With this retroviral backbone, huCD25 is also expressed to higher levels or antigen–antibody interactions are improved compared with our earlier work with HSA (23). To demonstrate the utility of this method, we have successfully enriched multiple transduced cell types. These include NIH 3T3 cells, Fabry mouse BMMCs, Jurkat cells, normal human MSCs, and simian virus 40-immortalized Fabry patient fibroblasts. Cells ranged from as low as 3–5% positive after infection to 80–95% positive after a single immunoaffinity isolation procedure (Fig. 1*A*). Yields of recovered huCD25$^+$ cells varied considerably depending on the type and number of cells to be enriched. MSC recovery was only $\approx$20% of initially positive cells. Approximately 50% of huCD25$^+$ fibroblasts were recovered whereas $\approx$75% of huCD25$^+$ murine BMMC and Fabry patient CD34$^+$ cells were recovered (data not shown).

Importantly, enrichment of transduced cells led to significantly higher levels of α-gal A activity over that in nonsorted cells *in vitro*. Enrichment of transduced normal human MSCs (from $\approx$19% to $\approx$82% huCD25$^+$) led to a 3-fold increase in intracellular and a 14-fold increase in secreted α-gal A activity. Results for simian virus 40-immortalized Fabry fibroblasts were even more striking, as enrichment (from $\approx$8% to $\approx$96% huCD25$^+$) led to intracellular α-gal A activity increases of 9-fold and secreted α-gal A activity increases of nearly 30-fold.

Enrichment of Transduced Fabry Patient CD34$^+$ Cells. Fabry patient CD34$^+$ hematopoietic cells are an important target for gene

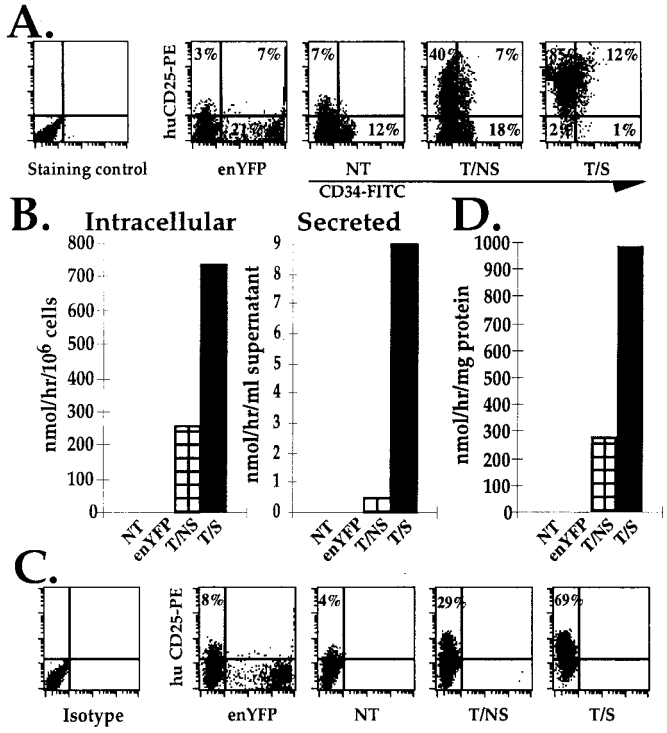

Fig. 2. Bulk immunoaffinity enrichment of transduced mobilized Fabry patient PB CD34$^+$ cells. (*A*) Flow cytometric analyses of enrichment. (*B*) Intracellular and secreted α-gal A activity after sorting. Flow cytometric analyses (*C*) and intracellular α-gal A activity (*D*) after 16 days of culture in supplemented methylcellulose.

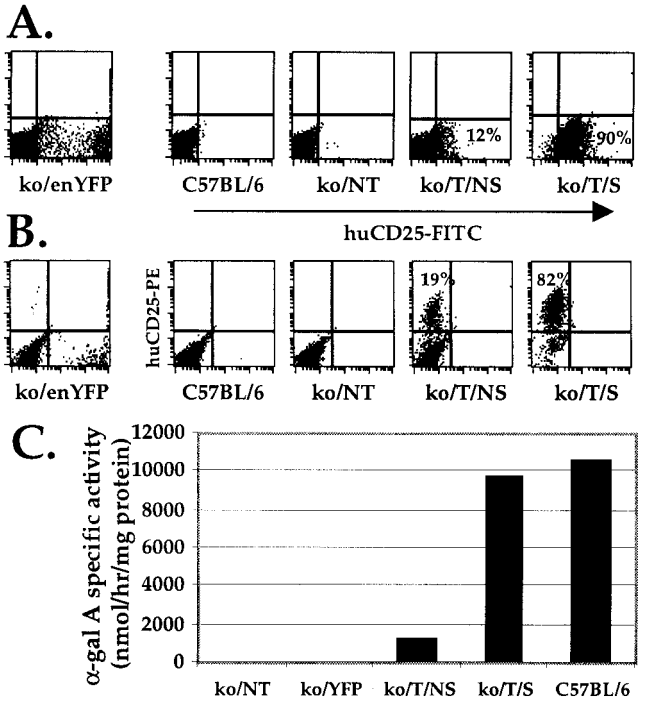

Fig. 3. Immunoaffinity enrichment of transduced Fabry mouse BMMC. (*A*) Flow cytometric analyses of enrichment. Flow cytometric analyses (*B*) and intracellular α-gal A activity (*C*) after 12 days of culture in supplemented methylcellulose.

therapy (7). To examine the effect of preselection on this population, mobilized Fabry patient PB CD34$^+$ cells were isolated, infected, and enriched. Staining of the freshly isolated cells showed ≈96% CD34$^+$, ≈35% CD4$^+$, ≈8% CD8$^+$, and negligible CD14$^+$ cells. Importantly, only ≈0.4% of cells were huCD25$^+$. After infection with the PG13-clone or control virus, expression of huCD25 was assessed. A representative result is shown in Fig. 2. In the control nontransduced cells (NT) and the enYFP-infected cells (enYFP), ≈10% of the CD34$^+$ cells expressed endogenous huCD25. In the therapeutically infected but nonsorted cells (T/NS), ≈47% of the population expressed huCD25. Positive cells could be enriched further (T/S population) to >95% purity by a single immunoaffinity sorting procedure (Fig. 2*A*). The T/S Fabry patient CD34$^+$ cells showed intracellular and secreted α-gal A activities that were increased 3-fold and 18-fold, respectively, compared with the T/NS cells (Fig. 2*B*). NT- and enYFP-transduced cells showed little α-gal A activity. Intracellular and secreted levels of GC were similar for T/S and T/NS cells (data not shown).

We then plated control and huCD25-enriched CD34$^+$ cells in methylcellulose. After 16 days, cells in the NT culture showed ≈4% huCD25$^+$ marking, the T/NS culture showed ≈29% huCD25$^+$ marking, and the T/S culture showed ≈69% huCD25$^+$ levels (Fig. 2*C*). The intracellular α-gal A activity of cells from the T/S culture was found to be 3.5-fold higher than the T/NS culture (Fig. 2*D*). The control NT and enYFP group showed minimal α-gal A activity. Intracellular GC activities were slightly reduced in the α-gal A-transduced colonies (data not shown). We also enumerated and sized colony types. There were no differences in these parameters between the NT CD34$^+$ cells and the T/NS or T/S CD34$^+$ cells (data not shown), suggesting that huCD25 overexpression in primitive human hematopoietic cells does not affect the colony-forming ability and quantity. Lineage-specific analysis of various populations also showed no obvious bias in hematopoietic cell differentiation between the different groups (data not shown).

Further Study of huCD25 Overexpression. We examined the effects of overexpression of huCD25 on transduced human cells by other methods. The fraction of transduced huCD25$^+$ cells did not decrease or increase with extended propagation *in vitro* in comparison with controls for all transduced cell lines (data not shown). We also studied the effect of overexpression and enrichment of huCD25 on human cell lines (Jurkat, HH, and HEL) in culture in the presence and absence of human IL-2. We examined cell proliferation with a [H^3]thymidine incorporation assay. Restoration of huCD25 expression to CD25-deficient HH cells led to a significant proliferative advantage; however, no proliferative advantage for any cell type was gained by the overexpression of huCD25 with any level of human IL-2 (data not shown).

Primary Transplanted Fabry Mice. We tested the effects of transplantation of preselected cells *in vivo* in the murine model of Fabry disease. We enriched transduced BMMC that were ≈12% positive to ≈90% positive (Fig. 3*A*), among which ≈40% are Sca-1$^+$, which encompasses primitive murine hematopoietic cells. We then plated Fabry mouse BMMC into methylcellulose cultures with cytokines for differentiation and marking studies. After 12 days, ≈82% of the total pooled colony cells were huCD25$^+$ in the T/S culture, whereas in the T/NS culture only ≈19% of the total cells were huCD25$^+$ (Fig. 3*B*). Intracellular α-gal A activity of pooled cells in the T/S culture reached values close to that observed in the wild-type (wt) culture and was 9-fold higher than cell from the T/NS culture (Fig. 3*C*). Colonies of different myeloid lineages (G, M, GM, GEMM) were represented equally in all groups, even though the level of huCD25 transgene expression in different lineages varied but was consistent between colonies derived from T/S and T/NS groups (data not shown).

We then infused Fabry mouse BMMC into lethally irradiated Fabry mice at a dose of 10^6 cells per mouse (except the low-dose

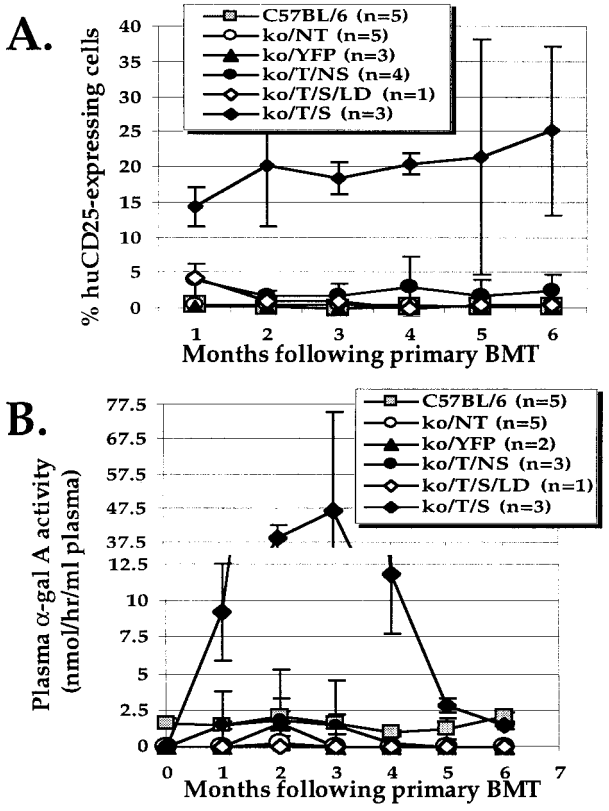

Fig. 4. PBMC huCD25 marking (*A*) and plasma α-gal A activity (*B*) in primary transplanted Fabry mice.

group, which received 10^5 cells per mouse) via tail-vein injection in the following groups: T/NS, T/S, low-dose sorted group (T/S/LD), and enYFP (nonsorted, ≈30% positive) group. We also maintained an NT and a wt group as controls. Initially, five animals comprised each group. After bone marrow transplantation (BMT), the mice were bled monthly to follow huCD25 expression on PBMCs and α-gal A activity levels in plasma. Engraftment was variable between the groups receiving transduced cells. For the T/S/LD group, only one of five animals engrafted (as evidenced by >1% huCD25$^+$ cells in PBMCs), and for the enYFP group, three animals died and two of two animals engrafted (data not shown). For the T/NS and T/S groups, three of five animals showed positive engraftment. Only engrafted animals in all groups were used in subsequent analyses. Over time, the T/S group collectively maintained a 10-fold-higher proportion of huCD25$^+$ cells in PBMCs than the T/NS group (Fig. 4*A*). These levels of marked cells were maintained for 6 months. The T/NS group was found to have slightly higher plasma α-gal A activity than the control wt group at 2 months (Fig. 4*B*). This decreased to slightly lower than the wt group at 3 months and continued to decline thereafter. The NT and enYFP groups showed minimal plasma α-gal A activity. Meanwhile, the T/S group showed a 19-fold-higher plasma α-gal A activity than the T/NS group at 2 months and nearly 30-times higher at 3 months (Fig. 4*B*). After 3 months, these values declined but were still similar to the wt group (and above the T/NS group, $P < 0.03$ for all months, single-tail t test) for the duration of the study. The T/S/LD group initially showed very low huCD25 staining in PBMCs and became negative from 2 months onward for all animals in that group.

We also stained PBMCs for lineage markers. All transduced and control groups had a similar composition of neutrophils (CD14), monocytes (CD11b), T cells (CD3), and B cells (CD19) in their PBMCs at months 4, 5, and 6 (data not shown). We then checked huCD25 expression in lineages. We found successful

	Heart	Kidney	Brain	Liver	Lung	Spleen
WT (n=5)	0.43±0.12	2.72±0.39	10.76±1.51	6.5±0.88	6.6±0.87	26.41±3.19
ko/NT (n=5)	0.01±0.01	0.04±0.01	0.04±0.02	0.02±0.02	0.16±0.18	0.31±0.12
ko/enYFP$^+$ (n=2)	0.00±0.00	0.04±0.01	0.02±0.00	0.03±0.00	0.04±0.01	0.21±0.13
ko/T/NS (n=3)	0.14±0.19	0.06±0.06	0.02±0.02	0.93±1.29	0.44±0.62	11.24±17.98
ko/T/S/LD (n=1)	0	0.04	0.029	0.14	0.05	0.35
ko/T/S (n=2)	0.60±0.22	0.20±0.11	0.20±0.11	4.29±0.72	3.86±2.36	58.58±30.88

Fig. 5. Organ α-gal A activities (nmol/hr per mg protein) of primary transplanted Fabry mice at 6 months.

marking of multiple lineages at months 4, 5, and 6 at a relatively high efficiency (up to 26%) in each animal receiving enriched cells (data not shown). The T/S group showed higher marking percentages than the T/NS group. The proportion of huCD25 marked cells was as follows: macrophage > T cells > B cells = granulocytes. This result was maintained over time. These findings indicate that preselection enriched successfully for transduced hematopoietic cells that have multilineage capability and that expression of huCD25 does not substantially influence murine reconstitutive hematopoiesis after BMT.

Mice were killed 6 months after primary BMT, and kidney, liver, heart, brain, lung, and spleen were obtained from each animal. GC enzyme activity levels were similar for each animal of each control and test group (data not shown). Fig. 5 shows the observed α-gal

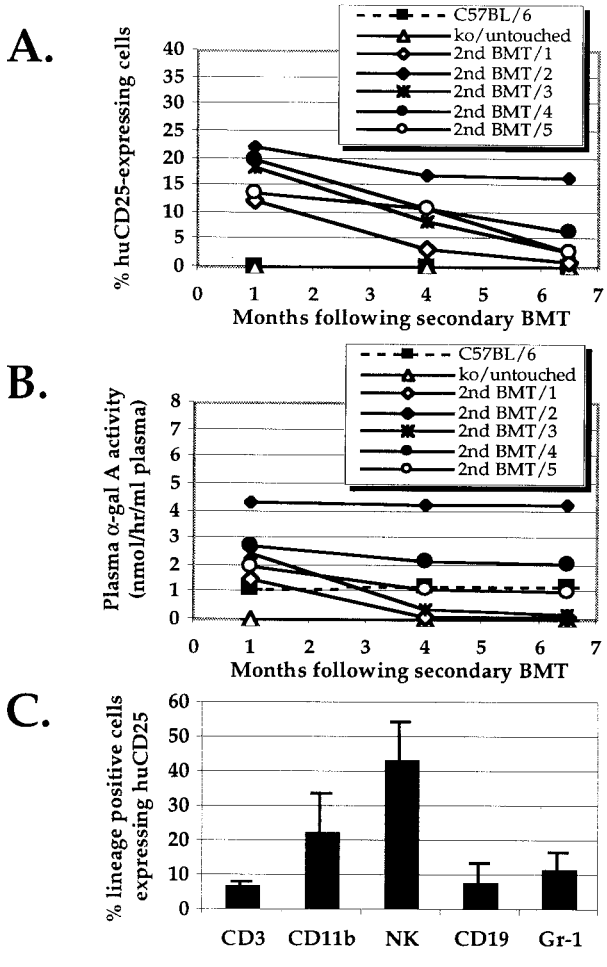

Fig. 6. PBMC huCD25 marking (*A*), plasma α-gal A activity (*B*), and hematopoietic lineage comarking (*C*) of secondary transplanted Fabry mice.

A-specific activity levels for each organ. Values for the enYFP group mirrored those from the NT group, as did the T/S/LD group. Transplantation of T/NS cells increased organ α-gal A activities over background (NT) as observed previously (9). Most striking, however, was that preselection followed by transplantation (T/S) led to statistically significant ($P < 0.05$) increases of α-gal A activity levels in all tissues (except kidney) over time compared with the T/NS group. Indeed, in contrast to our earlier work (9), even brain α-gal A activity was heightened. Lung and liver α-gal A activities significantly benefited from the enrichment procedure and more nearly approximated normal values when the T/S population was transplanted instead of the T/NS population. Lastly, heart and spleen showed the greatest increases and actually surpassed wt values at this 6-month time point after T/S cell transplantation.

Secondary Transplanted Fabry Mice. Simultaneously with organ harvests, BMMC were collected from the primary transplanted T/S group for secondary transplantations. Five lethally irradiated Fabry mice received 2×10^6 nonenriched cells that were $\approx$10% positive for huCD25 expression. PBMCs from secondary transplanted mice were analyzed for huCD25 expression at 1, 4, and 6.5 months posttransplantation (Fig. 6*A*). Cell surface expression of huCD25 ranged from $\approx$11–22% at month 1 and declined to $\approx$1–17% at 6.5 months. α-Gal A plasma expression levels were fairly stable over time (Fig. 6*B*) and correlated well with the number of huCD25$^+$ PBMCs. In two recipient animals, the levels of plasma α-gal A activity surpassed wt values. PBMCs from the secondary transplanted animals were stained for multiple hematopoietic lineages and huCD25 (Fig. 6*C*). huCD25 marking ranged from $<$10% of CD3$^+$ cells to $>$40% of NK$^+$ cells.

Discussion

If hematopoietic engraftment occurs in a limited "space" in recipients, then transplantation of a higher percentage of cells that are functionally corrected for a genetic defect should allow more effective competition for that space. Furthermore, if metabolic cooperativity is a function of the abundance of transduced cells, it would follow that therapeutic outcomes could be enhanced by employment of an *in vivo* or *ex vivo* selection strategy. In this study we sought to develop and test this premise in an animal model of Fabry disease. We observed that a variety of potential therapeutic cell targets can be enriched effectively and that this preselection technique significantly improves measurable outcomes *in vivo*.

Besides the fact that huCD25 is a human molecule, we chose to employ it in this system for a number of other reasons. It is encoded by a small cDNA and, thus, does not significantly affect viral titer (unlike *MDR*1). It has a low binding affinity for IL-2 and is nonsignaling by itself. It is a hematopoietic cell surface molecule and, therefore, may have less untoward effects on engraftment or other hematopoietic cell functions than other markers. It is not expressed at appreciable levels on a variety of noninfected target cells, yet appreciable levels are readily detected upon engineered overexpression. Furthermore, the structure of huCD25 is well studied, making it possible to make mutations to disrupt various moieties if necessary without affecting antibody binding. Because of the lack of cross-reactivity with murine antigens, we can also track the fate of transduced cells *in vivo* in combination with other techniques such as immunohistochemistry. Furthermore, we actually can begin to quantitate the number of circulating transduced cells that contribute to systemic α-gal A levels, thus giving defined target end points to be achieved for clinical implementation.

Preselection may also obviate another important limitation in the field of retrovirus-mediated gene transfer. Kalberer *et al.* (32) recently have shown that this enrichment approach may circumvent *in vivo* gene silencing and age-dependent extinction of expression. In this work, we observed multilineage marking of hematopoietic cells that persists over 6 months in secondary transplanted animals. In the interest of expanding this approach, we are also examining the use of other cell types or hematopoietic populations for this preselection approach. Further, we plan to examine cell doses and recipient ablation permutations for optimal correction in this model as well. *In vivo* studies also will be performed in transplanted Fabry mice with added human and murine IL-2.

One issue that remains is the recovery efficiency of transduced cells. Here, we observed recoveries of up to 75% of huCD25$^+$ cells. Further studies are underway to optimize recovery. For this approach to affect the clinical course of Fabry disease, it may be necessary to collect cells from multiple apheresis procedures before transduction and preselection. Recovered cells then could be isolated and reinfused incrementally over time to maintain appreciable circulating and elevated organ levels of α-gal A.

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