Preliminary Communication

Outcomes Following Gene Therapy in Patients With Severe Wiskott-Aldrich Syndrome

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IMPORTANCE Wiskott-Aldrich syndrome is a rare primary immunodeficiency associated with severe microthrombocytopenia. Partially HLA antigen–matched allogeneic hematopoietic stem cell (HSC) transplantation is often curative but is associated with significant comorbidity.

OBJECTIVE To assess the outcomes and safety of autologous HSC gene therapy in Wiskott-Aldrich syndrome.

DESIGN, SETTING, AND PARTICIPANTS Gene-corrected autologous HSCs were infused in 7 consecutive patients with severe Wiskott-Aldrich syndrome lacking HLA antigen–matched related or unrelated HSC donors (age range, 0.8-15.5 years; mean, 7 years) following myeloablative conditioning. Patients were enrolled in France and England and treated between December 2010 and January 2014. Follow-up of patients in this intermediate analysis ranged from 9 to 42 months.

INTERVENTION A single infusion of gene-modified $CD34^+$ cells with an advanced lentiviral vector.

MAIN OUTCOMES AND MEASURES Primary outcomes were improvement at 24 months in eczema, frequency and severity of infections, bleeding tendency, and autoimmunity and reduction in disease-related days of hospitalization. Secondary outcomes were improvement in immunological and hematological characteristics and evidence of safety through vector integration analysis.

RESULTS Six of the 7 patients were alive at the time of last follow-up (mean and median follow-up, 28 months and 27 months, respectively) and showed sustained clinical benefit. One patient died 7 months after treatment of preexisting drug-resistant herpes virus infection. Eczema and susceptibility to infections resolved in all 6 patients. Autoimmunity improved in 5 of 5 patients. No severe bleeding episodes were recorded after treatment, and at last follow-up, all 6 surviving patients were free of blood product support and thrombopoietic agonists. Hospitalization days were reduced from a median of 25 days during the 2 years before treatment to a median of 0 days during the 2 years after treatment. All 6 surviving patients exhibited high-level, stable engraftment of functionally corrected lymphoid cells. The degree of myeloid cell engraftment and of platelet reconstitution correlated with the dose of gene-corrected cells administered. No evidence of vector-related toxicity was observed clinically or by molecular analysis.

CONCLUSIONS AND RELEVANCE This study demonstrated the feasibility of the use of gene therapy in patients with Wiskott-Aldrich syndrome. Controlled trials with larger numbers of patients are necessary to assess long-term outcomes and safety.

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Wiskott-Aldrich syndrome (WAS) (OMIM 301000) is a complex X-linked primary immunodeficiency caused by loss-of-function mutations in the *WAS* gene. The condition affects the immunohematopoietic system and has a broad spectrum of severity.[1] The WAS protein (WASp) is a key regulator of the actin cytoskeleton in all hematopoietic lineages.[2] WASp deficiency causes characteristic microthrombocytopenia and lymphoid and myeloid cell dysfunction, the severity of which is usually correlated with WASp expression levels. A clinical scoring system is used to stratify disease severity.[3] Patients with a score from 3 to 5 display a WAS phenotype characterized by a tendency to bleed, persistent eczema, susceptibility to severe opportunistic bacterial and viral infections, autoimmune and inflammatory complications, and an elevated risk of lymphoid malignancies.[3-5] In the absence of definitive treatment, patients with classic WAS do not survive beyond their second or third decade of life. Although hematopoietic stem cell (HSC) transplantation is usually curative, the use of partially HLA antigen-matched HSCs is associated with a high incidence of complications.[6-10] Gene therapy based on transplantation of autologous, gene-corrected HSCs may be an effective and potentially safer alternative.

HSC *hematopoietic stem cell*

MPB *mobilized peripheral blood*

PCR *polymerase chain reaction*

PBMC *peripheral blood mononuclear cell*

WAS *Wiskott-Aldrich syndrome*

WASp *Wiskott-Aldrich syndrome protein*

The first gene therapy trial for WAS used a Moloney leukemia virus–derived γ-retroviral vector. Although this therapy provided significant clinical benefit characterized by partial or complete resolution of immunodeficiency, autoimmunity, and bleeding diathesis, it was associated with an unacceptably high risk of insertional mutagenesis, with activation of several proto-oncogenes leading to leukemia in 7 of the 9 evaluable patients.[11]

We developed and tested a self-inactivating lentiviral vector for WAS gene correction (referred to as LV-w1.6 WASp) in which a 1.6-kb fragment of the proximal promoter of the *WAS* gene is used to express the full-length coding sequence of the human *WAS* gene in cells of the hematopoietic lineage.[12-14] In a recently published study, 3 young children with a moderate form of WAS were treated with this vector. They showed stable engraftment of WASp-expressing cells and improvements in terms of immune function, platelet count, and clinical score.[15] Herein, we report the first results of a 2-center study designed to assess the feasibility of HSC gene therapy in patients with severe WAS.

Methods

Clinical Protocol

Seven consecutive patients with confirmed WAS were enrolled at Great Ormond Street Hospital (London, England) and Necker Children's Hospital (Paris, France) in an open-label study between December 2010 and January 2014. The dates of final follow-up were between May 28 and November 12, 2014.

The study protocol was approved by the UK and French drug regulatory agencies and the appropriate investigational review boards such as the Gene Therapy Advisory Committee in the United Kingdom and the Ethical Committee for the Protection of the Persons Submitted to a Clinical Trial. Written informed consent or assent was obtained after the benefits and risks of the trial were explained to the patients or their parents/legal guardians.

Hematopoietic stem cells were collected from patient bone marrow or mobilized peripheral blood (MPB) and were genetically modified ex vivo during myeloablative conditioning of patients. All patients were placed in sterile confinement and received a low-intensity conditioning regimen with busulfan (4 mg/kg/d) and fludarabine (40 mg/m^2/d) for 3 days. At the end of the conditioning procedure, transduced cells were infused to patients without cryopreservation. Anti-CD20 antibody and/or alemtuzumab were added if autoimmune disease was present. Autoimmunity was defined clinically as cutaneous or large vessel vasculitis, arthritis, and cytopenia of 1 or more hematopoietic lineages in association with the presence of autoantibodies. All transduced cell products met the specifications required for product release and infusion.

The synopsis of the study protocol is provided in eAppendix 1 of the Supplement.

Outcomes

Primary study objectives were to assess outcomes following gene therapy, judged by improvement in clinical manifestations including frequency and severity of infections, bleeding episodes, autoimmune manifestations, and eczema.

Secondary objectives were based on biological tests including platelet count, lymphocyte subset analysis, and lymphocyte function (mitogen- and antigen-induced proliferation, serum immunoglobulin levels). Secondary objectives of safety were evaluated clinically (including manifestations of clonal proliferation or leukemia) and by assessment of the frequency of vector integration sites close to relevant proto-oncogenes and their abundance within the engrafted and transduced cell population.

The severity of disease in patients was scored according to the following criteria[3,5]: A score of 1 accounts for microthrombocytopenia, which is universal to all patients; a score of 2 includes mild eczema, immunodeficiency, and occasional mild infections; a score of 3 refers to more severe immunodeficiency associated with recurrent and more protracted infections; a score of 4 is given if eczema or infections are persistent and do not respond easily to conventional treatments; and a score of 5 is assigned to very severe clinical forms that additionally develop autoimmune or malignant complications.

Vector Production

The LV-w1.6 WASp vector has been described elsewhere.[14,16] Clinical vector batches were manufactured at Genethon (Evry,

France) according to good manufacturing practices and were purified, concentrated, and titered for infectious particles (infectious genomes per milliliter).[16] The batches used in the study are described in eTable 1 in the Supplement. Batch 1 was used in London, and batches 2 and 3 were used in Paris.

Biological Analyses

Lymphocyte phenotypes, functions, and T-cell receptor repertoires (β, α, γ, and δ chains) were analyzed as described elsewhere.[17-20] Signal joint T-cell receptor excision circles were determined using real-time quantitative polymerase chain reaction (PCR).[21] T-cell receptor excision circle content was expressed in copies per 10^5 peripheral blood mononuclear cells (PBMCs) (control range, 150-2500/10^5 PBMCs). The vector copy number per cell was measured by quantitative PCR detection of the vector's human immunodeficiency virus psi sequence with normalization against the copy number of the albumin gene, as described elsewhere[22] (see description of methods in eAppendix 2 in the Supplement).

Lymphoid, myeloid, naive, and memory T-cell subpopulations were sorted by flow cytometry using the corresponding fluorescence-labeled monoclonal antibodies. Natural killer cell cytotoxicity was evaluated against K562 target cells as described in eAppendix 2.

CD34$^+$ Cell Gene Transfer Procedure

Patient bone marrow cells were harvested under general anesthesia, separated with lymphoprep (Eurobio), and centrifuged to collect mononuclear cells. Patient MPB was collected by apheresis. Positive selection of CD34$^+$ cells from mononuclear cells or from MPB was performed using immunomagnetic beads and an immunomagnetic enrichment device (CliniMACS, Miltenyi Biotec). Purified CD34$^+$ cells were seeded on cell culture bags precoated with clinical-grade RetroNectin (Takara Bio Inc) in serum-free medium (X-Vivo 20; Biowhittacker/Lonza) and clinical-grade stem cell factor (300 ng/mL), Fms-like tyrosine kinase 3 ligand (300 ng/mL), thrombopoietin (100 ng/mL), and IL-3 (20 ng/mL) (all from Peprotech). After 24 hours of prestimulation, cells were transduced twice with LV-w1.6 WASp (10^8 infectious genomes/mL), for 18 hours each time. At the end of the transduction procedure, washed cells were resuspended in 4% human serum albumin and transferred in a sterile bag for infusion to the patient. Aliquots of cells were further cultured for 14 days to measure stable proviral integration by quantitative PCR and WASp expression by flow cytometry.

Integration Site Analysis and Clonality Assays

Ligation-mediated PCR was used to sequence vector integration sites in different cell subpopulations (the cell selection procedure is described in eAppendix 2 in the Supplement).[23-25] At least 3 independent replicates were analyzed for all samples. Deep sequencing was carried out using both the 454/Roche and Illumina techniques. Data sets analyzed are summarized in eAppendix 3 in the Supplement. Assays of a DNA preparation were judged to be successful if they detected at least 80 different break sites in the human genome associated with unique adaptor ligation positions. All integration site sequence data were deposited at the National Center for Biotechnology Information Sequence Read Archive (SRP050221).

Statistical analysis was carried out using R software, version 3.1.2 (http://www.r-project.org). An extensive discussion of statistical methods and results is presented in eAppendix 3 in the Supplement. Briefly, the distributions of integration sites (relative to genomic features) were summarized using the receiver operating characteristic curve method (eFigure 1, A and B, in the Supplement).[26] The abundance of cell clones was quantified using the SonicLength method[25] and the Shannon index. The Shannon index is calculated as

$$H' = -\sum_{i=1}^{R} p_i \ln p_i$$

where p_i is the proportion of the cells (determined as fragment lengths) belonging to the ith integration site and R is richness. The Shannon index summarizes both the number of different unique integration sites and the evenness of distribution of cells harboring each unique site.

Clumping of integration site sequences was analyzed using scan statistics.[27]

Results

Clinical Presentation

At enrollment, the age of the patients ranged from 0.8 to 15.5 years (median, 7 years). All but 1 of the patients (patient 6) had a disease score of 5 (**Table 1**). All patients experienced severe thrombocytopenia, which led to severe bleeding episodes in patients 2, 3, 4, 5, and 7 (intracerebral hemorrhage in patient 3; gastrointestinal hemorrhage in patient 5). Furthermore, all patients had eczema and associated recurrent skin infections. Three of the 7 patients experienced recurrent, severe infections requiring hospitalization. Six patients had autoimmune disease; patients 2 and 3 were most profoundly affected. In patient 2, a combination of severe lower limb vasculitis and arthritis became refractory to conventional treatment and prevented ambulation.

Patient 3 had severe autoimmune cytopenias that led to splenectomy at age 3 years. He also experienced a lymphoproliferative disorder with generalized lymphadenopathy, liver enlargement, and renal infiltration. His primary immunodeficiency was responsible for severe infections of cytomegalovirus, herpes simplex virus type 1, and varicella zoster virus, which led to several hospitalizations from age 7 years to the time of gene therapy treatment including for perioral herpes infections with facial cellulitis and severe respiratory tract infection with *Klebsiella pneumoniae* aggravated by a cerebral hematoma causing coma and requiring intensive care unit hospitalization. He developed sequelae following his stroke requiring rehabilitation therapy. The recurrent herpes virus infections were treated with several cycles of acyclovir, ganciclovir, and foscarnet requiring hospitalization (Table 1). The patient's herpes simplex virus type 1 genotype was found resistant to acyclovir in the months that preceded gene therapy.

Table 1. Characteristics of Patients Before and After Gene Therapy[a]

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Clinical manifestations in the 2 y preceding gene therapy							
Bleeding[b]	Bruising	Severe Splenectomy	Severe Splenectomy Multiple platelet transfusions	Severe Multiple platelet transfusions	Severe Multiple platelet transfusions; N-plate	Bruising (moderate)	Severe Multiple platelet transfusions; N-plate
Eczema[c]	Severe	Severe	Mild	Mild	Severe	Severe	Severe
Infections[d]	Severe	2 episodes of sepsis, severe VZV	Severe chronic VZV, HSV, CMV, EBV infections	Mild	Mild	Severe	Gastroenteritis (2 episodes)
Autoimmunity	Recurrent arthritis, renal disease	Severe lower limb vasculitis and arthritis; unable to walk	Pancytopenia	Severe thrombocytopenia, mild skin vasculitis[e]	Severe thrombocytopenia[e]	None	Severe thrombocytopenia, mild skin vasculitis[e]
Time in hospital for disease-related complications	<5 d	47 d	>6 mo	26 d (in 10 mo)[f]	19 d	<5 d	21 d
Clinical disease score[g]	5	5	5	5	5	3	5
Date of gene therapy	March 3, 2011	May 27, 2011	November 18, 2011	April 20, 2012	June 15, 2012	November 16, 2012	January 24, 2014
Date of last follow-up	November 12, 2014	May 28, 2014	Death on July 11, 2012	October 4, 2014	June 11, 2014	November 12, 2014	November 3, 2014
Follow-up, mo	42	36	7	30	24	24	9
Clinical status 2 y after gene therapy or at last follow-up	Alive and well	Alive and well	Died of opportunistic viral infections	Alive and well	Alive and well	Alive and well	Alive and well
Bleeding	Bruising (resolved after splenectomy)	No	NA	Bruising	Bruising	Bruising (mild)	Bruising
Eczema[c]	No	Very mild	NA	No	No	No	No
Infections[d]	No	Localized zoster (1 episode)	NA	No	Mild gastroenteritis (1 episode)	No	No
Autoimmunity	No	Mild vasculitis, able to walk	NA	No	No	No	No
Days in hospital (after initial gene therapy period) over following 2 y	0	5	Continuous hospitalization until death	0	5	0	0
Clinical disease score at last follow-up	<1	5 (mild autoimmunity)	NA	<1	<1	<1	1

Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; NA, not applicable; VZV, varicella zoster virus.

[a] Patients 1 and 6 were included in London and patients 2, 3, 4, 5, and 7 were included in Paris.

[b] Platelet count was initially below 20 000/μL in patients 1, 2, 3, and 6 and below 10 000/μL in patients 4, 5, and 7. Patients 2 and 3 underwent splenectomy prior to gene therapy. Patient 1 had a successful splenectomy after gene therapy. Patients 3, 4, and 5 had more than 20 platelet transfusions, patient 7 had more than 10, and patients 5 and 7 received additional N-plate therapy.

[c] Severe eczema is defined by intense redness and thickness/swelling and may include diffuse or superinfected lesions.

[d] Severe infection indicates infection requiring hospitalization and parenteral treatment.

[e] Severe thrombocytopenia is defined as platelet count below 10 000/μL.

[f] Patient 4 had a shorter period of observation since he was treated at the age of 10 months.

[g] Patients with a score from 3 to 5 display a WAS phenotype characterized by a tendency to bleed, persistent eczema, susceptibility to severe opportunistic bacterial and viral infections, autoimmune and inflammatory complications, and an elevated risk of lymphoid malignancies.

Glossary

Complementarity-determining regions, or hypervariable regions, are regions that confer the specificity to a given T-cell receptor. These regions are short segments of about 10 amino acids in the variable domains of T-cell receptor chains in which lie the greatest part of the variability between different T-cell receptors. There are 3 complementarity-determining regions in each chain of the T-cell receptor α or β chains. These complementarity-determining regions adopt loop structures that, when combined, form a surface complementary to the 3-dimensional structure of the antigen bound.

Lentiviral vectors are nonreplicative hybrid viral particles designed for gene transfer into dividing and nondividing cells. Lentiviral vectors use elements derived from human immunodeficiency virus type 1 to stably integrate their therapeutic gene expression cassette into the genome of target cells in a semirandom manner. Lentiviral vectors are used in gene therapy for gene transfer into hematopoietic stem cells, T cells, neurons, or retinal cells.

Mobilized peripheral blood is a convenient source of hematopoietic stem cells, which are mobilized from the bone marrow niches to the peripheral blood by repeated (ie, once daily for 5 days) administration of granulocyte colony-stimulating factor. Mobilized peripheral blood cells are then collected by apheresis and $CD34^+$ stem cells are purified from mobilized peripheral blood by magnetic cell sorting.

Revertant cells recovered some expression from the originally mutated gene as a result of selected spontaneous somatic mutations.

T-cell receptor excision circles are extrachromosomal (nonreplicative) DNA byproducts of T-cell receptor rearrangement. These episomes are found only in naive T cells, which contain a single copy of T-cell receptor excision circles. Hence, T-cell receptor excision circle analysis by quantitative polymerase chain reaction is used to evaluate thymic output and function. It provides a very specific assessment of T-cell recovery (eg, after hematopoietic stem cell allogeneic transplantation or after hematopoietic stem cell gene therapy).

Vector copy numbers are defined by the amount of vector-specific sequences amplified from a population of cells in relation to the amount of cellular genes, as calibrated against a standard sequence, and expressed in copies per cell based on 2 copies of the cellular gene (albumin) per cell. The measure of gene marking in various blood cell subsets by evaluation of mean vector copy numbers per cell is widely used in the field of gene therapy.

Gene Transfer

Autologous $CD34^+$ HSCs (from bone marrow in 4 cases and from MPB in 3 cases; **Table 2**) were transduced ex vivo with the LV-w1.6 WASp lentiviral vector and were immediately reinfused into conditioned patients. The median dose of $CD34^+$ cells per kilogram of body weight infused was 7.3×10^6 (range, 2×10^6 to 15×10^6) and the mean vector copy number per cell in $CD34^+$ cells was 1.27 (SD, 0.8; range, 0.6-2.8) (Table 2).

Outcomes After Gene Therapy

Six of the 7 patients treated with gene therapy were evaluable over a period of at least 9 months (mean and median follow-up, 28 and 27 months, respectively) for primary outcomes.

Patient 3 died as a consequence of opportunistic herpes viral infections that became drug resistant after gene therapy, including severe perioral necrotizing ulcerative lesions caused by herpes simplex virus type 1 and bilateral cytomegalovirus retinitis with high blood viral load (4.6 log copies/mL) (eTable 2 in the Supplement). This was associated with an inflammatory pulmonary syndrome characterized by multiple foci of bronchoalveolar parenchymal condensation responsible for oxygen dependence developed in association with diffuse *Aspergillus*-related lesions. The patient's clinical state rapidly worsened. A lung biopsy showed an extensive fibrosis. The patient eventually died of septic shock in the intensive care unit 7 months after gene therapy.

In December 2014, the other 6 treated patients were alive and showed significant clinical improvements (Table 1). In terms of the primary outcomes, eczema and susceptibility to infections had resolved in all cases. Minor, nonrecurrent infections were observed in 2 patients (Table 1). Patient 1 had not had any further episodes of arthritis. Patient 2 showed major improvement in peripheral vasculitis and was able to return to normal physical activity without need for a wheelchair. Patient 7 recovered completely from vasculitis skin lesions. With the exception of occasional bruising, there were no posttreatment recurrences of the severe, recurrent bleeding episodes that had previously affected patients 2, 4, 5, and 7. From month 7 onward, none of the 6 surviving patients required regular blood product support or treatment with recombinant stimulators of platelet production (patient 4 received N-plate until month 13).

Days of additional disease-related hospitalization once the initial gene therapy was completed were reduced to 0 to 5 days over the next 2 years for patients 1, 2, 4, 5, and 6 and over a 9-month period for patient 7. Patient 1 had a 7-day hospitalization for elective splenectomy in year 3 after engraftment. After gene therapy, patients continued their pretreatment regimen of immunoglobulin substitution and prophylactic antibiotics, although immunoglobulins were recently discontinued in patients 4 and 6 pending evaluation of functional responses to a typical childhood vaccination schedule.

Gene Marking and WASp Expression in Leukocytes

The presence of vector-positive cells in blood was readily detectable 1 month after treatment. Quantitative PCR revealed an increase over time in gene marking in PBMCs (**Figure 1**). Gene marking in PBMCs was primarily due to transduced lymphocytes, most prominently T cells, but multiple cell lineages were also transduced. At the last follow-up, the vector copy number per cell ranged from 0.35 to 1.20 in sorted $CD3^+$ T cells, from 0.1 to 1.34 in sorted $CD19^+$ B cells, and from 0.04 to 0.7 in sorted natural killer $CD56^+$ cells. The extent of gene marking in $CD14^+$ or $CD15^+$ myeloid cells was more variable, with 0.4 copies per cell in patient 4, 0.2 copies per cell in patient 6, 0.1 copies per cell in patients 2 and 7, and between 0.01 and 0.03 copies per cell in patients 1 and 5 (Figure 1).

The intracellular expression of WASp correlated with the levels of transduction in the different cell subsets. At the last follow-up, the proportion of cells expressing WASp was highest

Table 2. Characteristics of Infused Autologous Gene Therapy Products[a]

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age at time of gene therapy	10 y	15.5 y	10 y	10 mo	3 y	7 y	3.5 y
WAS gene mutation	401C/T p.A134V	c.1453+1 G>C	c.628delT p.S210HfsX51	c.1295delG p.G432EfsX13	c.257G>A p.R86H	100C>T p.R34X	1271dup(ex10) Leu425Profsx70
Revertant cells[b]	–	+	+	–	–	+	–
Mutant WASp expression[b]	+	–	–	–	–	–	+
Hematopoietic stem cell origin	BM	MPB	MPB	BM	BM	BM	MPB
Total CD34$^+$ cell dose, x10^6/kg	2	11	11	7.3	6.8	3.1	15
Vector copy number per cell[c]	0.7	1.3	1.2	2.8	0.6	1.7	0.6

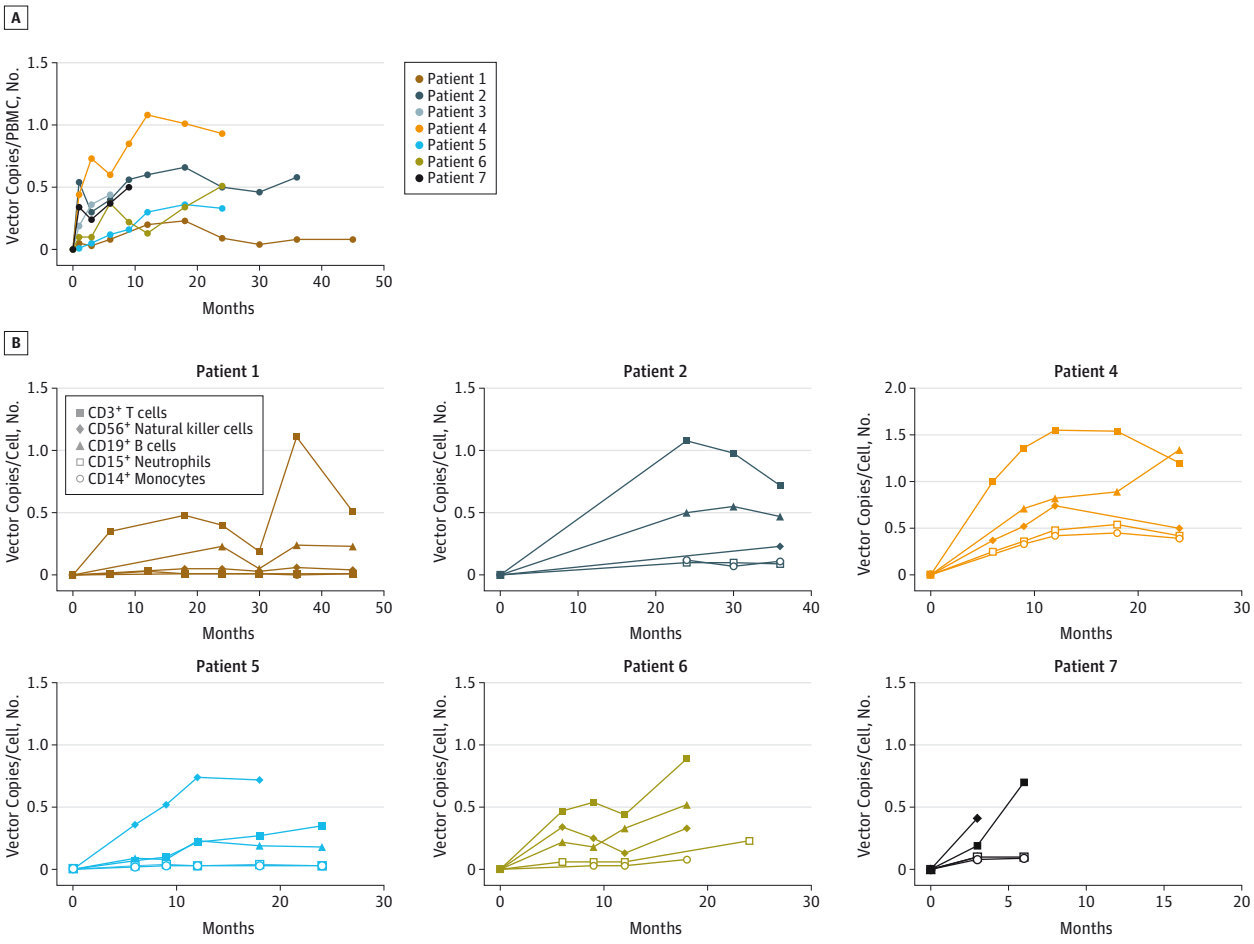
Abbreviations: BM, bone marrow; MPB, mobilized peripheral blood; WAS, Wiskott-Aldrich syndrome; WASp, Wiskott-Aldrich syndrome protein.

[a] Patients 1 and 6 were included in London and patients 2, 3, 4, 5, and 7 were included in Paris.

[b] Plus signs indicate that revertant cells or mutant protein were present and detectable in blood cells . Minus signs indicate that revertant cells or mutant protein were absent and not detectable in blood cells.

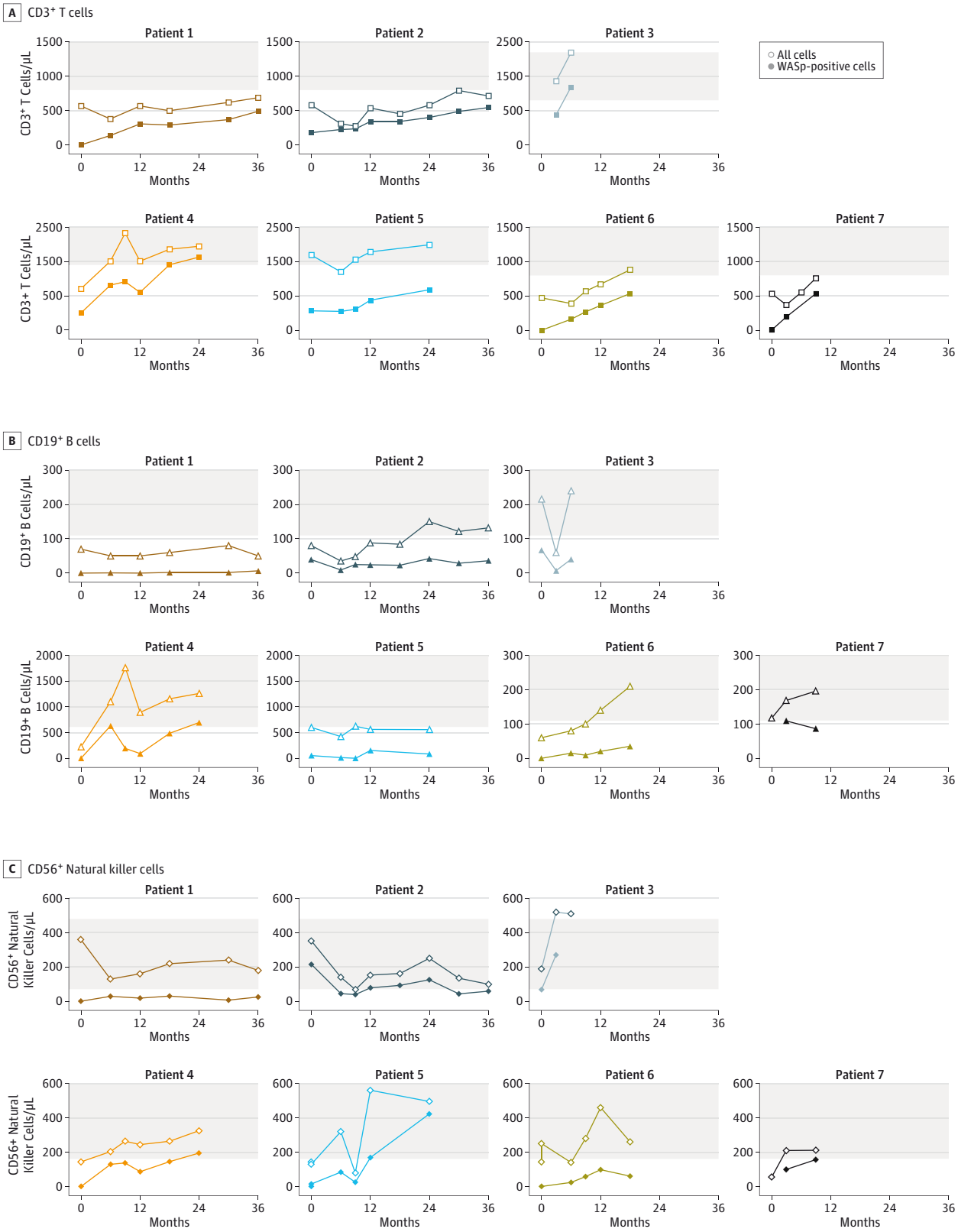
[c] In cultured transduced CD34$^+$ cells.

Figure 1. Longitudinal Evaluation of Gene Marking in Blood Cells After Gene Therapy



A, Gene marking in peripheral blood cells over time after gene therapy in patients 1 to 7, as expressed by vector copy number per peripheral blood mononuclear cell (PBMC) and measured by quantitative polymerase chain reaction. B, Gene marking in various blood cell subsets in each patient, expressed as vector copy number per cell in CD3$^+$ T cells, CD56$^+$ natural killer cells, CD19$^+$ B cells, CD15$^+$ neutrophils, and CD14$^+$ monocytes.

Figure 2. Time Course of Lymphoid Cell Subset Recovery in Blood After Gene Therapy



The total and Wiskott-Aldrich syndrome protein (WASp)–positive levels of CD3+ T cells, CD19+ B cells, and CD56+ natural killer cells were measured in blood over time. Tinted areas indicate values in aged-matched individuals.

in T cells (34%-84%) and somewhat lower in natural killer cells (14%-85%) and B cells (13%-55%) (**Figure 2**, **Figure 3**, and **Table 3**).

Immune Reconstitution

Recovery of normal absolute T-cell counts was achieved in 4 of the 6 evaluable patients (patients 1, 4, 5, and 6), while values remained just below the normal range in patients 2 and 7 (Table 3 and Figure 2). Normal counts of $CD4^+$ T cells were demonstrated in the same 4 patients. Low absolute $CD8^+$ T-cell counts were observed in 4 of 6 patients (Table 3 and eFigure 2 in the Supplement).

In all patients, ongoing thymopoiesis was evidenced by recovery of circulating naive T cells and detection of T-cell receptor excision circles. One patient had normal absolute naive $CD4^+$ T-cell counts; for the other 5 of 6 evaluable patients they remained below the normal range (Table 3 and eFigure 2).

For patient 3, the follow-up period was too short to evaluate immune reconstitution, although T-cell numbers recovered rapidly after treatment and were transgene positive prior to his death 7 months after gene therapy. The presence of anticytomegalovirus-specific T cells was detected by pp65 cytomegalovirus-specific tetramers as well as interferon γ^+ immunostaining (eTable 2 in the Supplement). This is an evidence of partial, albeit functionally incomplete, specific T-cell response.

Expression of functional WASp is known to provide T cells with a significant growth and survival advantage; this is especially true for the memory subset and effector populations that normally express higher levels of WASp than naive T cells.[29-31] Accordingly, vector copy numbers were repeatedly found to be higher in memory T cells than in naive T cells in patient 4 (12 months after gene therapy, we found 2.1 vs 1.1 vector copies per cell, respectively). Although WASp is not essential for natural regulatory T-cell generation, it is required for its function.[32,33] After gene therapy, $CD4^+/CD25^+/FoxP3^+$ regulatory T cells expressed the *WAS* transgene in all 3 patients evaluated (patients 2, 4, and 5) (Figure 3).

At last follow-up, absolute natural killer cell counts were within the normal range in all patients and functioning was normal in the sample available for testing (patient 7) (Figure 2, Table 3, and eFigure 3 in the Supplement). At last follow-up, B-cell counts were within the normal range in 3 of the 6 patients (patients 2, 4, and 6) (Figure 2 and Table 3).

Immunoscope analysis revealed that the transduced T cells displayed a polyclonal T-cell receptor repertoire (eFigure 4 in the Supplement). As a consequence of impaired T-cell receptor–mediated signaling, T cells from patients with WAS typically show defective proliferation after stimulation with anti-CD3 monoclonal antibodies.[34-36] After gene therapy, all patients had normal proliferative responses to phytohemagglutinin, and anti-CD3 antibody–mediated proliferation was also observed in all patients except patient 1 (Table 3). A positive response to 1 or more microbial antigens (tetanus toxoid, varicella zoster virus, *Candida albicans*, and purified protein derivative) was detected in patients 2, 4, 5, and 7 (Table 3).

WASp-deficient dendritic cells and monocytes fail to form the actin-rich adhesion structures known as podosomes.[37,38] After gene therapy, monocyte-derived dendritic cells from patients 2, 4, and 5 were assayed for their ability to assemble podosomes on adhesion to fibronectin. The fraction of podosome-positive dendritic cells in patients 4, 2, and 5 was 50%, 20%, and less than 5%, respectively, demonstrating that WASp expression (which was correlated with myeloid cell gene marking) restored the dendritic cells' ability to regulate cytoskeletal rearrangements.

As of December 2014, B-cell function could not be fully evaluated because 4 of 6 patients were still receiving immunoglobulin replacement therapy, but 2 patients (patients 4 and 6) had recently stopped immunoglobulin treatment. In patient 4, the analysis of the B-cell phenotype 24 months after gene therapy showed B-cell subsets within normal ranges compared with healthy age-matched reference values, probably due to WASp expression restoration in B cells[39] (eFigure 5 in the Supplement). Moreover, serum IgE levels, which are generally elevated in patients with WAS, decreased significantly in patient 2 and become very low in patient 7 (eTable 3 in the Supplement).

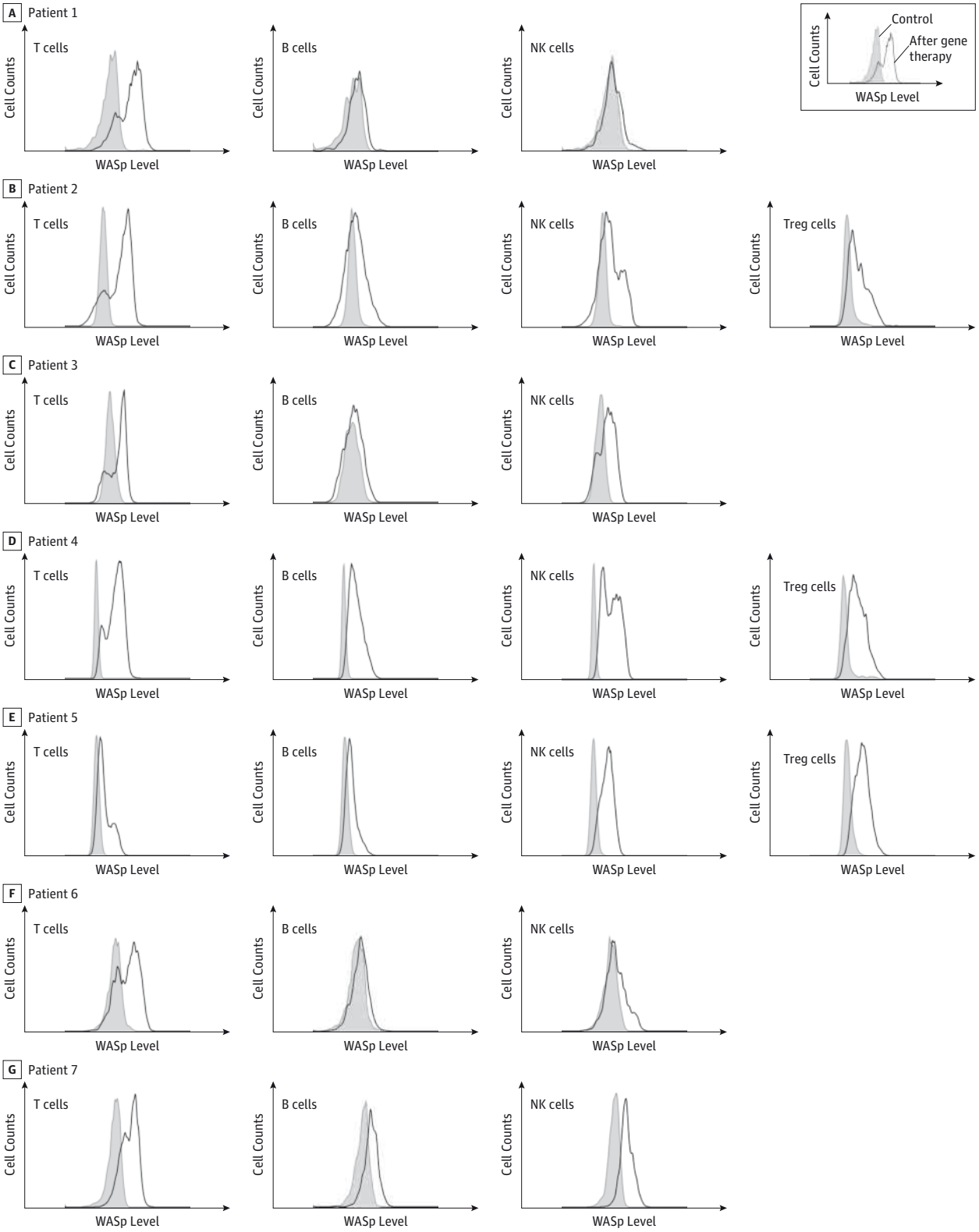
Platelet Reconstitution

Platelets counts and platelet volumes were variably ameliorated by gene therapy in the 6 evaluable patients (Table 3 and **Figure 4**A). Although all patients continued to have thrombocytopenia, the detected platelets were predominantly WASp positive (Figure 4B). The patients' mean platelet volume (range, 8.1-9.3 fL) was in the normal range for healthy controls in our centers (7.5-10.1 fL). Patients receiving the highest doses of transduced $CD34^+$ cells appeared to exhibit the most pronounced increases in platelet counts, but findings were not statistically tested (Table 2, Table 3, Figure 4A, and **Figure 5**). Patient 1 underwent splenectomy for quality-of-life reasons 3 years after gene therapy and recovered normal platelet counts.

Analysis of Vector Integration Site Distribution

More than 5 million genomic integration site sequence reads were collected from sorted cell samples from the 6 patients, yielding more than 90 000 unique integration sites. As expected for a lentiviral vector, correlation of the integration site distribution with genomic features revealed preferential integration within transcription units and association with epigenetic markers of transcription in the infused gene therapy products (pretransplantation) and in circulating blood cells at different time points after gene therapy (posttransplantation)[23,40] (eFigure 1, A and B, in the Supplement). Analysis of genomic regions with high frequencies of integration revealed clusters of sites[27] in both the pretransplantation and posttransplantation samples (also referred to as "clumps" in eAppendix 3). Clusters were spread over large genomic distances (>0.5 Mb) and did not overlap with the 5′ ends of the genes implicated in the occurrence of adverse events in previous gene therapy trials (ie, *LMO2*, *CCND2*, and *MECOM*/*EVI1*) (eFigure 1, C and D, and eFigure 6 in the Supplement).

Figure 3. WASp Expression by Flow Cytometry Using Fluorescent-Labeled Monoclonal Antibodies in Lymphoid Cell Subpopulations at Different Times After Gene Therapy



WASp marking in the T cells, B cells, and natural killer (NK) cells of patients 1 to 7 performed respectively at months 30, 30, 6, 24, 24, 12, and 9 are shown. WASp marking in the regulatory T (Treg) cells of patients 2, 4, and 5 were performed respectively at months 30, 18, and 18.

Table 3. Data From Most Recent Immunological Analysis of Patients

Characteristics	Patient 1	Patient 2	Patient 3[a]	Patient 4	Patient 5	Patient 6	Patient 7	Control Range, y	
								<7	≥7
Age at last follow up, y	14	18	11	3	5	9	4.3		
Time of analysis, mo after gene therapy	42	36	6	30	24	24	9		
$CD3^+$ T cells, /μL	840	715	2106	1921	1984	970	756	1400-3700	800-2200
$WASp^+$ T cells, %	71[b]	76	54	84[b]	34	61[b]	70	100	100
$CD4^+$ T cells, /μL	560	440	972	1256	1271	730	434	700-2200	530-1300
$CD8^+$ T cells, /μL	180	187	1053	471	620	180	196	490-1300	330-920
Naive $CD4^+$ T cells, %	37	23	1	27[b]	32[b]	54	22	60-72	43-55
Naive $CD8^+$ T cells, %	33	17	0.3	38[b]	15[b]	70	22	52-68	52-68
T-cell receptor excision circles, /10^5 PBMCs	847[b]	612[b]	ND	1118[b]	1444[b]	130[b]	ND	>150	>150
Phytohemagglutinin-induced proliferation	C	C	C	C	C	C	C	C	C
Anti-CD3–induced proliferation	+/−[c]	C	ND	C	C	C	C	C	C
Antigen-induced proliferation	ND	2[d]	0	1[b,d]	1[d]	ND	1[d]	NA	NA
$CD56^+$ natural killer cells, /μL	190	99	297	324[b]	496	240	210	160-950	70-480
$WASp^+$ natural killer cells, %	14[b]	60	ND	60[b]	85	23	74	100	100
$CD19^+$ B cells, /μL	70	132	270	1260[b]	558	170	196	610-2600	110-570
$WASp^+$ B cells, %	13[b]	27	17	55[b]	15	15[b]	44	100	100
Platelets, x10^9/L	217[e]	87	71[f]	44	6	13	10	>100	>100
Platelet volume, mean, fL	9.1[e]	7.8[b]	ND	9.1[b]	ND	9.1	ND	5-12	6.5-12
$WASp^+$ platelets	ND	C	ND	C	C	ND	ND	C	C

Abbreviations: C, comparable to control range; NA, not applicable; ND, not determined; PBMC, peripheral blood mononuclear cell; WASp, Wiskott-Aldrich syndrome protein.

[a] Patient 3 died 7 months after gene therapy of opportunistic infections.

[b] Measured at the previous time point.

[c] Proliferation below the normal value.

[d] Positive T-cell proliferation with any of the following antigens tested (tetanus toxoid, varicella zoster virus, candidin, and purified protein derivative), numbers indicate the number of antigen to which the patient's cells proliferated.

[e] Measured at month 42, after splenectomy. Control values for lymphocytes are from Shearer et al.[28] The standards for the other measures have been established by local hospital diagnostic laboratories.

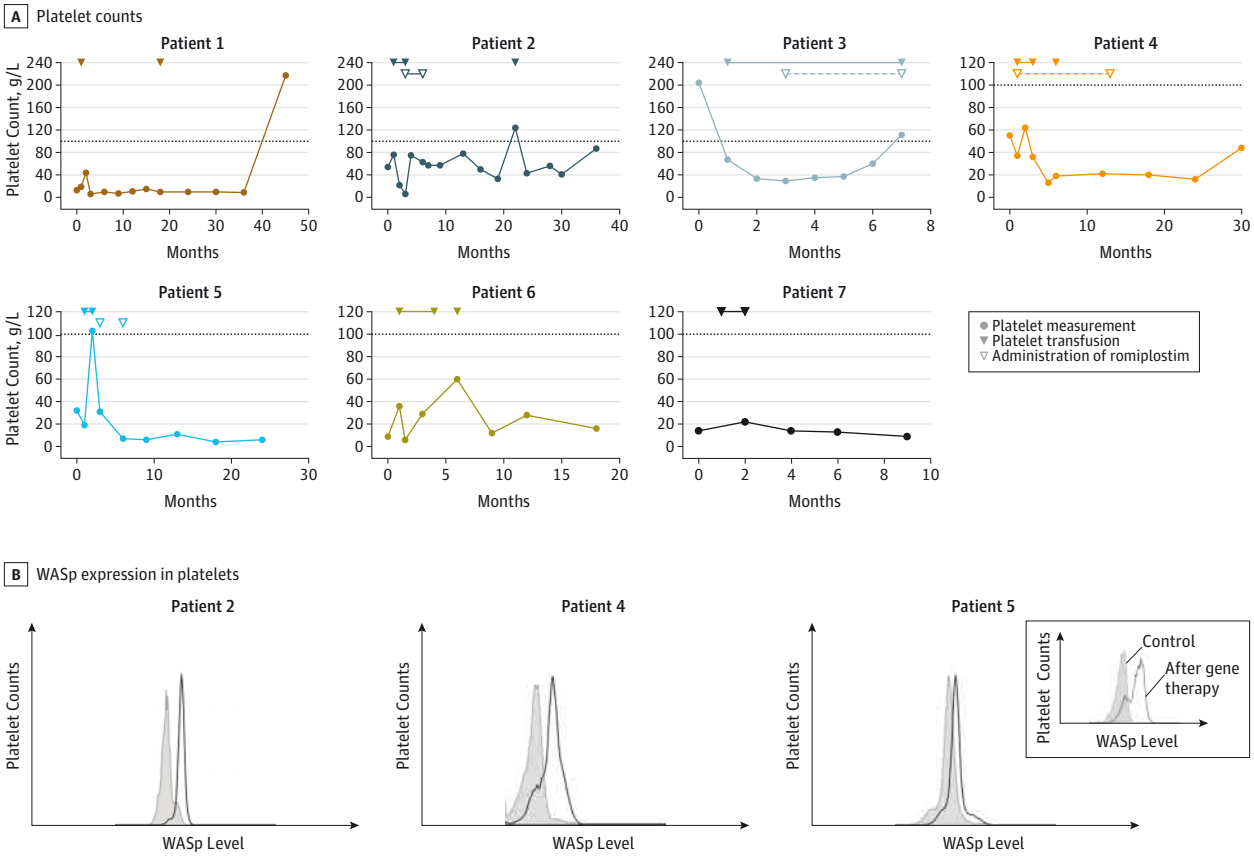
[f] During platelet transfusion.

Gene-rich regions showed more clusters, occasionally overlapping with cancer-associated genes in some patients at statistically nonsignificant frequency. Clones associated with genes of concern were a far smaller proportion of sites in the WAS lentiviral trial than in the WAS gammaretroviral trial (~0.06% vs ~2%, respectively; $P < 10^{-6}$), and none showed persistent expansion over time (eFigure 7 in the Supplement) (for further analysis, see eAppendixes 2 and 3). Thus, in contrast to what has been observed in patients treated with gammaretroviral vectors,[11,41,42] we did not detect an association between recurrent integration near specific cancer-associated genes and cell amplification or persistence.

Sequencing of pretransplantation integration sites from a sample of the infused cells revealed up to 2.9×10^4 unique sites. Replicate analyses of pretransplantation samples from each patient showed relatively little overlap in the integration patterns, which is consistent with very large population sizes. Change in clonality of PBMCs and also in sorted peripheral blood lymphoid cells ($CD3^+$ T cells, $CD19^+$ B cells, and $CD56^+$ natural killer cells) and myeloid cells ($CD15^+$ neutrophils and $CD14^+$ monocytes) were monitored over time in the 6 evaluable patients (eFigure 7 in the Supplement). Population sizes in well-characterized samples could be modeled by comparison with the overlap in sites detected in replicate analyses (using the Chao 1 estimator with jackknife correction, as applied to counts of linker positions).[25] The estimated population sizes in PBMCs corresponded to hundreds or thousands of clones. In all analyzed cell types and in all patients, no one clone accounted for more than 10% of the population detected at a given time point. All patients had a stable, high-diversity Shannon index (eFigure 1E). These data indicate that reconstitution with gene-corrected cells was highly polyclonal, with intermittent progenitor activity and no lasting clonal expansion.

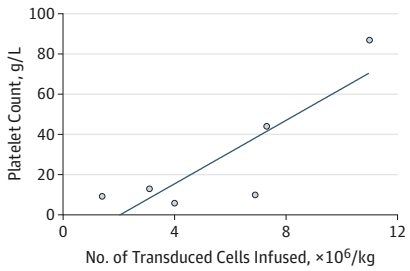
Common integration sites in myeloid and lymphoid lineages are indicative of the transduction of multipotent progenitors. We focused our analysis on highly pure (>97%)

Figure 4. Platelet Reconstitution After Gene Therapy



A, Change over time in platelet count in each patient. The lower normal value is indicated by a dotted line. Solid lines connecting the triangles indicate continuous platelet transfusions; dashed lines, continuous administration of romiplostim. B, Wiskott-Aldrich syndrome protein (WASp) expression in platelets, as measured by flow cytometry.

Figure 5. Correlation Between Platelet Reconstitution and Number of Transduced Cells Infused



The platelet count at last follow-up (Table 3) was plotted against the number of transduced CD34⁺ cells infused per kilogram of body weight, which was calculated by taking account of vector copy number values below 1.0 in the infused product (Table 2).

neutrophils and T and B lymphocytes sorted from patient 4 12 months after transplantation (eFigure 8 in the Supplement). These neutrophils shared 12% and 14% of their integration sites with T and B lymphocytes, respectively, suggesting that common progenitors were successfully transduced and engrafted in this patient.

Discussion

Wiskott-Aldrich syndrome is a multifaceted disease with a broad spectrum of severity.[1] There is still a need for novel, effective, well-tolerated treatments, particularly in patients with advanced disease and/or who lack an HLA-matched allogeneic donor.[10,43] This study reports the outcome of HSC gene therapy in 7 severely affected patients with WAS, using a lentiviral vector to transfer a WAS expression cassette in repopulating HSCs.[13,14] The protocol incorporated a near-myeloablative and immunosuppressive conditioning regimen to enhance the engraftment of transduced cells.

Compared with a recently reported monocentric study of 3 patients with WAS aged 1 to 6 years (and with a clinical score of 3 or 4),[15] the children in the present 2-center study were older and had more severe disease (with a clinical score of 5 among 6 of the 7 cases, which is a risk factor for allogeneic HSC transplantation). Among 6 of the 7 patients, there was clinical improvement after gene therapy, which was well tolerated. However, 1 patient died of preexisting, treatment-refractory infectious disease. In the 6 surviving patients, the infectious complications resolved after gene

therapy, and prophylactic antibiotic therapy was successfully discontinued in 3 cases. Severe eczema resolved in all affected patients, as did signs and symptoms of autoimmunity. No patient developed hemorrhagic complications after withdrawal of supportive treatment where implemented. T cell–related function was corrected in all evaluable patients, regardless of their age at the time of treatment or the dose of transduced cells received. A longer-term follow-up will be required to assess the functional reconstitution of humoral immunity, although the evidence of an accumulation of WASp-expressing B cells is encouraging.

Part of this study's objectives was to evaluate the hematological and immunological outcomes after engraftment of gene-corrected cells in various lineages. In all patients, the degree of gene marking in lymphoid cells was greater than that achieved in myeloid cells. This is consistent with a strong proliferative and/or survival advantage conferred on the lymphoid compartment by WASp expression, as predicted from earlier observations in mice and in cells from patients.[29,30] This hierarchy in gene marking has also been observed in patients included in 2 other gene therapy trials[11,15] and is in keeping with the results of previous studies of WASp status in patients after allogeneic HSC transplantation.

One of the major, invariable signs of WAS is a tendency to bleed as a result of intrinsic microthrombocytopenia, particularly when compounded by the development of antiplatelet antibodies. Platelet counts and mean platelet volume increased in 3 of the 6 evaluable patients (patients 2, 4, and 5) but remained below normal values, although no patient experienced any major bleeding episodes after gene therapy. Persistent thrombocytopenia also occurs after allogeneic HSC transplantation when associated with low myeloid chimerism.[10] The platelet counts measured in our patients and in other individuals treated with lentiviral gene therapy[15] are at the low end of the range observed in patients with mixed chimerism after allogeneic HSC transplantation. Recovery of platelet counts may be related to the dose of transduced cells received. These findings suggest that robust engraftment of HSCs is required to fully correct the disease phenotype. Nevertheless, lower-level engraftment enables lymphoid reconstitution as a consequence of the profound selective advantage conferred on these lineages.[30] Of note, a rapid normalization of platelet counts and platelet volume was obtained in patient 1 following splenectomy 45 months after gene therapy.

The present study is the first to our knowledge to demonstrate clinical improvement after autologous gene therapy using a lentiviral vector in severely affected children and young adult patients in whom more pronounced procedure-related complications would be expected. When considered alongside another study in younger and less severely affected patients,[15] this lentiviral vector may represent a safer alternative to a Moloney leukemia virus–derived vector used in a recently reported trial, in which 7 of the 9 patients developed acute leukemia as a result of insertional oncogenesis.[11]

Interpretation of the results of this type of study is constrained by the small number of patients and the difficulty in performing randomized trials in severe orphan diseases. We therefore cannot draw conclusions on long-term outcomes and safety. Further follow-up of these patients and those reported in a similar study last year,[15] together with additional clinical trials of this therapy, are therefore necessary.

Conclusions

This study demonstrated the feasibility of the use of gene therapy in patients with WAS. Controlled trials with larger numbers of patients are necessary to assess long-term outcomes and safety.

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