Evidence for Long-term Efficacy and Safety of Gene Therapy for Wiskott–Aldrich Syndrome in Preclinical Models

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Wiskott–Aldrich Syndrome (WAS) is a life-threatening X-linked disease characterized by immunodeficiency, thrombocytopenia, autoimmunity, and malignancies. Gene therapy could represent a therapeutic option for patients lacking a suitable bone marrow (BM) donor. In this study, we analyzed the long-term outcome of WAS gene therapy mediated by a clinically compatible lentiviral vector (LV) in a large cohort of *was*^{null} mice. We demonstrated stable and full donor engraftment and Wiskott–Aldrich Syndrome protein (WASP) expression in various hematopoietic lineages, up to 12 months after gene therapy. Importantly, we observed a selective advantage for T and B lymphocytes expressing transgenic WASP. T-cell receptor (TCR)-driven T-cell activation, as well as B-cell's ability to migrate in response to CXCL13, was fully restored. Safety was evaluated throughout the long-term follow-up of primary and secondary recipients of WAS gene therapy. WAS gene therapy did not affect the lifespan of treated animals. Both hematopoietic and non-hematopoietic tumors arose, but we excluded the association with gene therapy in all cases. Demonstration of long-term efficacy and safety of WAS gene therapy mediated by a clinically applicable LV is a key step toward the implementation of a gene therapy clinical trial for WAS.

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INTRODUCTION

Wiskott–Aldrich Syndrome (WAS) is a monogenic X-linked immunodeficiency also characterized by microthrombocytopenia, eczema, and a high susceptibility to develop tumors and multiple autoimmune manifestations.[1,2] WAS is caused by mutations in the *WAS* gene,[3] which impairs or abolishes the expression of the Wiskott–Aldrich Syndrome protein (WASP). WASP is expressed in hematopoietic cells,[4] where it integrates several extracellular stimuli to control actin cytoskeleton reorganization[5] and signal transduction.[6] WASP deficiency impairs several immune cell functions such as *in vivo* leukocyte migration,[7] pathogen killing by natural killer cells[8] and neutrophils,[9] antigen presentation by antigen-presenting cells,[10] homing of B cells to secondary lymphoid organs leading to dysfunctional humoral responses,[11] immunological synapse formation and T-cell activation after T-cell receptor (TCR)-engagement.[6,12–15] Thus, the wide range of hematopoietic cell types affected by the absence of WASP indicates that a valid therapeutic approach should target hematopoietic stem cells (HSCs).

Currently, the only resolutive therapeutic option for WAS patients is bone marrow transplantation (BMT) from related human leukocyte antigen–identical or matched unrelated donor.[16–18] Because it is crucial to proceed with BMT before the progressive worsening of the clinical status,[19] patients lacking a related identical donor or a matched unrelated donor often undergo BMT from a mismatched related donor. However, mismatched related donor transplantation is associated with an elevated risk of developing life-threatening Epstein–Barr virus lymphoproliferative syndrome, infections, autoimmunity, graft rejection, and graft-versus-host disease[16] resulting in reduced survival.[16–18] In the past few years, retroviral vector–mediated gene therapy emerged as a valid therapeutic alternative for patients with primary immunodeficiencies that could not benefit from conventional therapies.[20,21] Unfortunately, the occurrence of hematopoietic malignancies or preleukemic clonal expansions due to insertional mutagenesis was observed in clinical gene therapy trials for X-linked severe combined immunodeficiency and X-linked chronic granulomatous disease, respectively,[22] indicating the need of safer tools for gene transfer. Compared to the retroviral vectors used in the first clinical studies, lentiviral vectors (LVs) transduce nondividing HSCs

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effectively,[23] lack transcriptionally active long terminal repeats, and are less genotoxic.[24] We previously developed a human *WAS* promoter/cDNA encoding LV (w1.6W) that was successfully used to restore WASP expression in CD34$^+$ HSCs, T cells, B cells, and dendritic cells, and to correct TCR-driven activation in T-cell lines derived from WAS patients.[13,25] Moreover, the *in vivo* efficacy of this vector was demonstrated in a gene therapy setting in nonlethally irradiated *was*^{null} mice, with evidence of multilineage WASP expression in hematopoietic cells and correction of TCR-driven activation of splenic T cells.[26] However, gene therapy treated mice were followed up for 4 months, a period that is insufficient to investigate the long-term stability of WASP expression and T-cell functional correction, and the safety of the gene therapy approach. Indeed, it has been shown that severe adverse events related to gene therapy in mice may develop after a follow-up >7 months,[27,28] or after secondary BMTs.[29]

In this study, we analyzed the stability of WASP expression and the persistence of T- and B-cell functional correction in a large cohort ($n = 68$) of *was*^{null} mice treated with w1.6W-mediated gene therapy and followed up for 12 months. Safety was assessed by long-term monitoring of tumor incidence in primary ($n = 68$) and secondary ($n = 32$) recipients of WAS gene therapy, using two distinct models of *was*^{null} mice. We found that the w1.6W vector promoted long-term WASP expression in many hematopoietic cell lineages, which was associated to the selective advantage of WASP$^+$ T and B lymphocytes. In addition, WAS gene therapy corrected T-cell activation defects, normalized B-cell migration in response to CXCL13, and did not cause any severe adverse event. These results are instrumental for the implementation of an LV-mediated gene therapy clinical trial in WAS patients.

RESULTS

Transplantation of LV-transduced lin$^-$ *was*^{null} cells

To investigate the long-term efficacy and safety of WAS gene therapy in mice, we purified lineage marker–depleted (lin$^-$) cells from bone marrow (BM) BL6-*was*^{null} mice, and transduced them *ex vivo* by a single hit of a human *WAS* promoter/cDNA containing LV (w1.6W), at either low (10–20) or high (200) multiplicity of infection (MOI). Untransduced *was*^{null} and wt lin$^-$ cells were used as control (lin$^-$ *was*^{null} and lin$^-$ wt groups, respectively). The w1.6W LV integrated into *was*^{null} lin$^-$ cells and promoted the expression of transgenic WASP in a dose-dependent fashion (**Figure 1a**). Transduced or control lin$^-$ cells were injected in sex- or CD45 allele-mismatched BL6-*was*^{null} mice conditioned by sublethal irradiation. Donor cell engraftment was analyzed 1 year after transplantation by Y-chromosome specific real-time PCR (**Figure 1b**), or by flow cytometric detection of the CD45.2 allele (**Figure 1c**). High and stable engraftment (ranging 69–100%) was achieved in all hematopoietic cell types isolated from mice belonging to all experimental groups. These results demonstrate that sublethal irradiation of the recipient *was*^{null} mice is sufficient to ensure robust and persistent donor cell engraftment.

Analysis of long-term WASP expression in multiple hematopoietic cell lineages

To assess whether differentiated hematopoietic cells from gene therapy treated *was*^{null} mice expressed WASP 12 months after

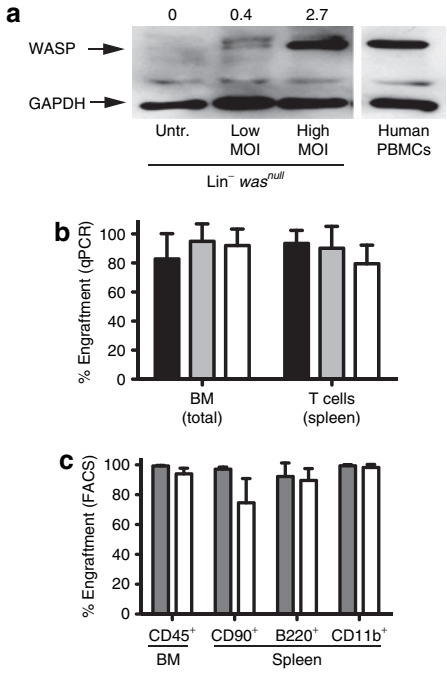

Figure 1 Transplantation of w1.6W-transduced lin$^-$ *was*^{null} cells for long-term experiments. (**a**) Western blot analysis of WASP expression in lysates of lin$^-$ *was*^{null} cells either untransduced or transduced with the w1.6W vector at low or high MOI. As control, WASP expression in human PBMCs is depicted. Numbers indicate VCN. (**b**) Donor cell engraftment measured by real-time PCR in the indicated cell types, 12 months after transplantation of transduced or control lin$^-$ cells. Mean ± SD is depicted. Lin$^-$ wt $n = 8$, black bars; low MOI $n = 11$, light grey bars; lin$^-$ *was*^{null} $n = 4$, empty bars. (**c**) Donor cell engraftment measured by flow cytometry analysis, 12 months after transplantation of transduced or control lin$^-$ cells. Mean ± SD is depicted. High MOI $n = 17$, dark grey bars; lin$^-$ *was*^{null} $n = 4$, empty bars. MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; VCN, vector copy number; WASP, Wiskott–Aldrich Syndrome protein.

treatment, we performed flow cytometric analysis. We detected WASP expression in BM CD45$^+$ cells, and in splenic myeloid cells (CD11b$^+$), B cells (B220$^+$), CD8$^+$ T cells, and CD4$^+$ T cells (**Figure 2a**), thus demonstrating effective long-term multilineage activity of the w1.6W vector *in vivo*. In all cell types analyzed, the proportion of WASP-expressing cells was higher in the high MOI group, as compared to the low MOI group (**Figure 2b,c**). Interestingly, in both MOI groups, the proportion of WASP-expressing splenic B cells, CD8$^+$ T cells, and CD4$^+$ T cells was significantly higher than that of BM CD45$^+$ cells and splenic myeloid cells (**Figure 2b,c**). Consistently, vector copy number (VCN) in splenic donor T cells was significantly higher than VCN in total donor BM cells, in both the low MOI (2.0 ± 0.8 versus 1.0 ± 0.4, $P < 0.05$) and the high MOI gene therapy group (4.2 ± 0.9 versus 2.7 ± 0.7, $P < 0.05$). Taken together, these data demonstrate the long-term expression of transgenic WASP in multiple cell lineages and strongly suggest the occurrence of a selective advantage for WASP-expressing lymphocytes.

Complete blood cell counts 12 months after gene therapy

Twelve-months-old BL6-*was*^{null} mice display B lymphopenia, thrombocytopenia, reduced monocyte counts, and modest

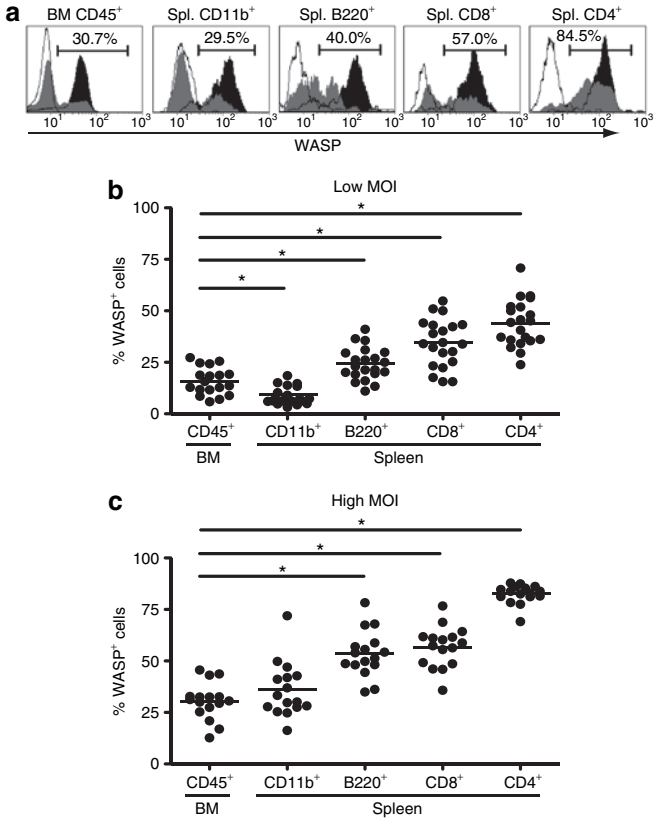

Figure 2 WASP expression in hematopoietic cells 12 months after gene therapy. (**a**) Analysis of WASP expression in BM CD45$^+$ cells, and splenic CD11b$^+$, B220$^+$, CD8$^+$, and CD4$^+$ cells. Histograms report WASP expression in a representative *was*^{null} (white), wt (black) and high MOI gene therapy treated mouse (gray). Numbers represent the percentage of WASP$^+$ cells in the high MOI gene therapy treated mouse. (**b**,**c**) Cumulative analysis of the percentage of WASP$^+$ cells in the indicated cell types within the low MOI gene therapy group (**b**, $n = 21$) or the high MOI gene therapy group (**c**, $n = 16$). Dots represent values from each single mouse. Bars indicate the mean value of the distribution. *$P < 0.05$, Student *t*-test. MOI, multiplicity of infection; WASP, Wiskott–Aldrich Syndrome protein.

granulocytosis (Table 1). To assess whether WAS gene therapy could restore normal hematopoietic cell counts, we performed complete blood cell counts on gene therapy treated animals and controls. Despite very high donor engraftment (Figure 1b), leukocyte, erythrocyte, and platelet counts in the lin$^-$ wt group were lower than those in nonirradiated wt mice (Table 1). Therefore, to evaluate the efficacy of WAS gene therapy, we took as reference the groups transplanted with wt or *was*^{null} lin$^-$ cells. The absolute count of T cells, B cells, and platelets in the lin$^-$ *was*^{null} group was decreased as compared to the lin$^-$ wt group, although granulocyte count was increased (Table 1). Mice treated with high vector MOI had B cell, platelet, and granulocyte counts comparable to those of the lin$^-$ wt group and significantly different from those of lin$^-$ *was*^{null} group. Conversely, mice belonging to the low vector MOI group had B cell, platelet, and granulocyte counts significantly different from those of the lin$^-$ wt group and similar to those of lin$^-$ *was*^{null} group (Table 1). T-cell counts in the lin$^-$ wt group were increased above levels of nonirradiated wt mice, although in the other groups they were comparable to levels found in nonirradiated *was*^{null} mice. Taken together, these data suggest that high MOI gene therapy could be more efficacious than low MOI gene therapy in ameliorating B cell, platelet, and granulocyte counts.

Long-term correction of T-cell activation after WAS gene therapy

The main consequence of WASP deficiency in T cells is impaired proliferation and cytokine production in response to TCR triggering.[6,13–15,30,31] After *in vitro* TCR stimulation, the proliferative response and the secretion of interleukin (IL)-2, interferon-γ, tumor necrosis factor-α, IL-4, and IL-10 were strongly reduced in *was*^{null} T cells, as compared to wt T cells (Figure 3a–f). WAS gene therapy performed at both high and low MOI completely corrected these defects, while transplantation of untransduced lin$^-$ *was*^{null} cells was ineffective (Figure 3a–f). The degree of functional correction was independent of the MOI used for initial transduction of lin$^-$ *was*^{null} cells. This indicates that WASP expression in 34–44% of splenic T cells (as found in the low MOI group, see Figure 2b) is sufficient to correct *was*^{null} T-cell dysfunctions. These data are in agreement with those observed in short-term gene therapy studies in *was*^{null} mice,[26,31–33] and further extend the efficacy of WAS gene therapy to a much longer time frame. Moreover, we provide the first demonstration that WAS gene therapy can restore Th2 cytokine secretion by T cells stimulated through the TCR.

Long-term correction of B-cell migration after WAS gene therapy

B lymphocytes isolated from *was*^{null} mice display impaired chemotaxis *in vitro*. This defect may contribute to decreased homing to B-cell areas of the spleen and to defective humoral immune responses *in vivo*.[11] To investigate the long-term functional correction of B cells after gene therapy, we measured migration of purified splenic B cells in response to the chemokine CXCL13 using a transwell system. Basal B-cell migration was comparable in all experimental groups (Figure 4). When exposed to CXCL13, the percentage of migrating wt B cells was 34.0 ± 5.2%, while it was only 23.0 ± 5.1% for *was*^{null} B cells (Figure 4). After WAS gene therapy performed at low MOI, migration of B cells in response to CXCL13 was normalized (Figure 4). This finding provides evidence of long-term efficacy of WAS gene therapy in restoring B-cell migratory potential.

Twelve-month follow-up of gene therapy treated mice

To evaluate safety of the WAS gene therapy approach, we periodically checked the general health status of WAS gene therapy treated BL6-*was*^{null} mice and controls. Survival curves of animals belonging to the high and low MOI groups were statistically similar to those of control lin$^-$ *was*^{null} and lin$^-$ wt groups, thus excluding a detrimental impact of lentiviral transduction and *WAS* transgene expression on overall survival (Figure 5a).

Evaluation of tumor occurrence could be performed in 32/34 mice in the lin$^-$ *was*^{null} group (94%), in 46/48 mice belonging to the low MOI gene therapy group (96%), in 18/20 mice in the high MOI gene therapy group (90%), and in 15/15 mice in the lin$^-$ wt group (100%). Overall tumor incidence 12 months after treatment was comparable in the gene therapy and control groups (Figure 5b). The occurrence of nonhematopoietic tumors (gut adenocarcinoma,

Table 1 Blood cell counts 12 months after gene therapy

Group	*n*	WBC (×10⁶/ml)[a]	T cells (×10⁶/ml)[a]	B cells (×10⁶/ml)[a]	Monocytes (×10⁶/ml)[a]	Granulocytes (×10⁶/ml)[a]	RBC (×10⁹/ml)[a]	PLT (×10⁶/ml)[a]
Wt	13	11.3 ± 2.3	2.0 ± 0.7	5.6 ± 1.8	1.0 ± 0.2	1.7 ± 0.9	10.0 ± 0.8	1,304 ± 214
was^{null}	11	6.6 ± 2.0[b]	1.5 ± 0.5	1.2 ± 0.7[b]	0.5 ± 0.2[b]	2.8 ± 1.4[b]	9.8 ± 1.1	655 ± 154[b]
Lin⁻ wt	13	7.4 ± 2.9[b]	2.9 ± 1.1[b,c]	1.8 ± 0.9[b,c]	0.5 ± 0.2[b]	1.6 ± 0.8[c]	9.1 ± 0.7[b]	941 ± 189[b,c]
High MOI	17	5.3 ± 2.4[b,d]	1.2 ± 0.6[b,d,e]	1.8 ± 0.7[b,c,e]	0.6 ± 0.4[b]	1.1 ± 1.0[c,e]	8.3 ± 1.8[b]	803 ± 220[b,c,e]
Low MOI	38	5.7 ± 1.9[b]	1.7 ± 0.6[d]	1.0 ± 0.5[b,d]	0.5 ± 0.2[b]	2.3 ± 1.3[d]	9.3 ± 1.1[b]	551 ± 134[b,d]
Lin⁻*was*^{null}	20	6.8 ± 3.2[b]	1.4 ± 0.7[b,d]	1.0 ± 0.6[b,d]	0.7 ± 0.5	3.2 ± 2.5[b,d]	9.1 ± 0.8[b]	614 ± 214[b,d]

Abbreviations: MOI, multiplicity of infection; RBC, red blood cells; PLT, platelets; WBC, white blood cells.
[a]Average value ± SD. [b]$P < 0.05$ as compared to wt. [c]$P < 0.05$ as compared to lin⁻*was*^{null}. [d]$P < 0.05$ as compared to lin⁻ wt. [e]$P < 0.05$ as compared to low MOI.

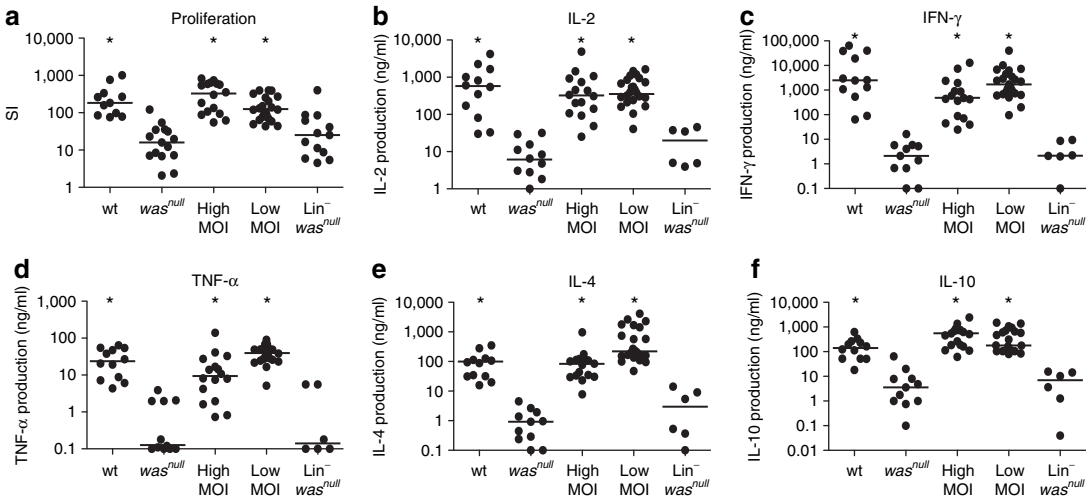

Figure 3 Long-term restoration of T-cell function after gene therapy. Splenic T cells were purified by immunomagnetic beads, and stimulated with 2 µg/ml anti-CD3 mAbs for 48 hours. (**a**) Proliferation was measured by ^{3}H-thymidine incorporation. wt $n = 11$; *was*^{null} $n = 15$; high MOI $n = 16$; low MOI $n = 23$; lin⁻ *was*^{null} $n = 13$. Results are expressed by the stimulation index (SI), *i.e.*, the ratio between cpm of stimulated and nonstimulated cells. (**b**–**e**) Cytokine levels were measured by BioPlex technology in conditioned supernatants. wt $n = 12$; *was*^{null} $n = 11$; high MOI $n = 16$; low MOI $n = 23$; lin⁻ *was*^{null} $n = 6$. Dots represent the measurement performed in each single mouse. Bars represent the median value. *$P < 0.05$ as compared to *was*^{null} group, Mann–Whitney test. cpm, counts per minute; MOI, multiplicity of infection.

hepatocarcinoma, ovary tumor, poorly differentiated thoracic carcinoma, eye carcinoma, skin carcinoma) was observed in four mice belonging to the lin⁻ *was*^{null} group (12.5%), three mice from the high MOI gene therapy group (16.7%), four mice from the low MOI gene therapy group (8.7%), and three mice (20.0%) belonging to the lin⁻ wt group (**Figure 5b** and **Supplementary Table S1**). As these tumors were of nonhematopoietic origin, it could be excluded that they were caused by gene therapy. Lymphomas were observed in two mice belonging to the lin⁻ *was*^{null} group (6.3%), and in four mice (8.7%) of the low MOI gene therapy group (**Figure 5b** and **Supplementary Table S1**). In all cases, real-time PCR analysis proved that they were of host origin and that they lacked LV integrations, thus excluding a role of gene therapy in the genesis of those hematopoietic malignancies (**Table 2**). In conclusion, our data generated in a large cohort of gene therapy treated mice followed up for 12 months strongly support the safety of the proposed gene therapy approach.

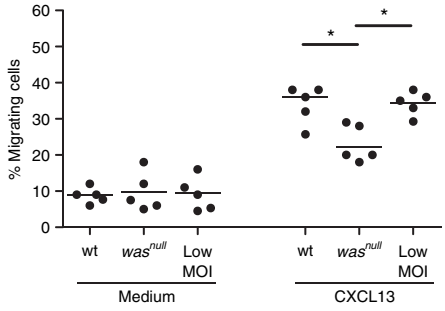

Figure 4 Long-term correction of B-cell migration after gene therapy. B cells were purified from the spleen of gene therapy treated or control mice. Migration through 5-µm pore transwells was measured in response to medium only or medium supplemented with 1 µg/ml CXCL13. Results are expressed as the percentage of input cells that migrated to the lower well. Each dot represents a single mouse. Bars depict the mean value. $n = 5$ per group. *$P < 0.05$ as compared to the *was*^{null} group, Student *t*-test.

Evaluation of safety of WAS gene therapy through serial BMT

To complete our safety evaluation, we performed secondary transplantations of w1.6W-transduced BM cells using a different model of WAS gene therapy based on the 129-*was*^{null} mouse strain and lethal irradiation.[32] Lin⁻ cells purified from 129-*was*^{null} mice were transduced with one or two hits of the w1.6W LV (WA 1X and WA 2X groups, respectively), or with one hit of pGFP control

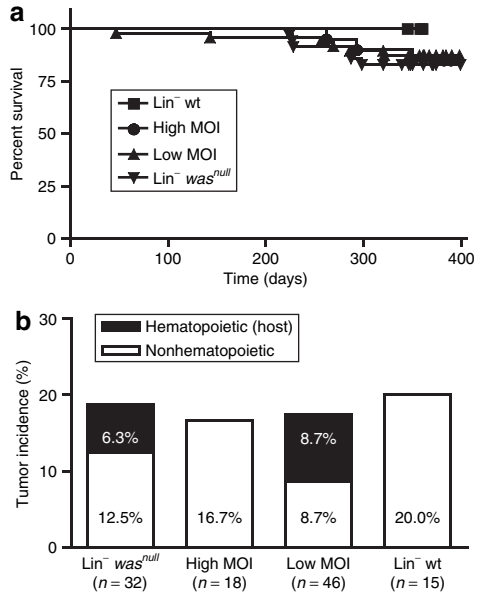

Figure 5 Twelve-month follow-up of gene therapy treated mice. (**a**) Survival curve of mice belonging to the lin⁻ wt (filled squares, n = 15), high MOI (filled circles, n = 20), low MOI (filled triangles, n = 48), and lin⁻ *was*^{null} (filled inverted triangles, n = 34) groups. The log-rank test failed to detect any statistically significant difference between the different groups. (**b**) Incidence of tumors in mice treated with gene therapy as compared to control groups. White bars represent the percentage of nonhematopoietic tumors, while black bars represent the percentage of host-derived hematopoietic tumors. Note that donor-derived hematopoietic tumors were never observed. Numbers represent the percentage of tumor incidence. MOI, multiplicity of infection.

vector (*was*^{null} GFP group). As further control, lin⁻ wt cells were transduced with one hit of pGFP vector (wt GFP group). Efficient w1.6W transduction of lin⁻ cells was demonstrated by high VCN and transgenic WASP expression (**Figure 6a**). Transduced lin⁻ cells were transplanted in lethally irradiated 129-*was*^{null} mice (11–12 per group, see **Supplementary Table S2**). Four months later, engraftment and median VCN of donor cells in the BM was comparable in all groups (**Figure 6b,c**). No tumor occurrence was observed in any recipient of primary graft (**Supplementary Table S2**).

To perform secondary transplantations, we selected 2–3 primary transplanted mice for each group, among those with the highest donor chimerism and/or VCN (**Figure 6b,c**). Two million total BM cells were transplanted in lethally irradiated, secondary 129-*was*^{null} recipients (5–6 for each donor mouse, 11–16 per group, see **Supplementary Table S3**). Secondary transplanted mice were followed up for 6 months. At killing, chimerism in the BM and spleen was variable but comparable to that in the BM of primary recipients (**Figure 6b**). VCN in the BM and spleen of secondary recipients was comparable to that in the BM of primary recipients in all groups except the WA 1X group, where a decreased VCN was observed in the BM only (**Figure 6c**). *WAS* mRNA expression was detected in the BM and spleen of mice belonging to the WA 1X and WA 2X groups (data not shown). Taken together, these results indicate that w1.6W-transduced HSCs engrafted in secondary recipients. Multilineage hematopoietic cell reconstitution in all groups was demonstrated by hemogram analysis (**Supplementary Table S3**)

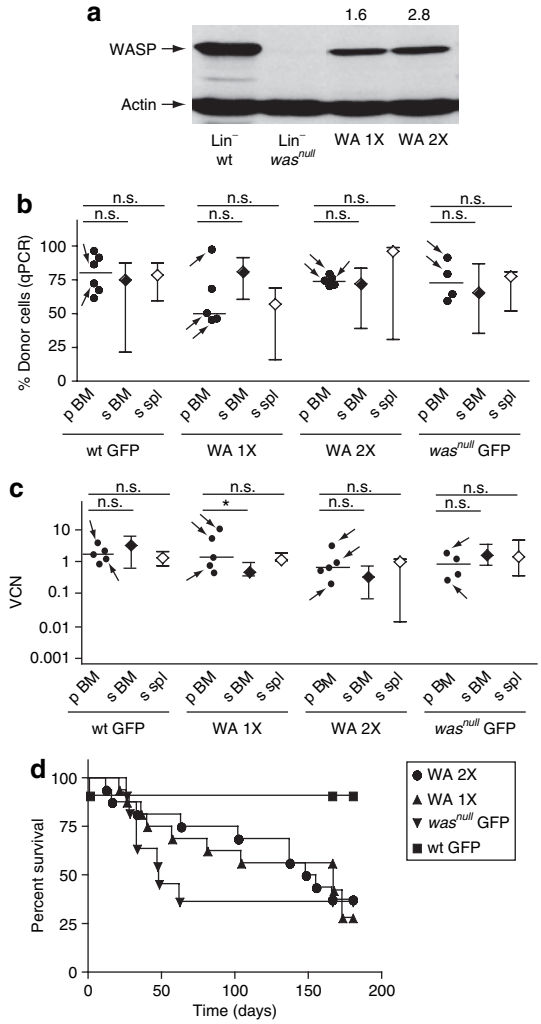

Figure 6 Follow-up of mice recipient of secondary bone marrow transplantation. (**a**) Western blot analysis of WASP expression in lysates of 129-*was*^{null} lin⁻ cells either untransduced or transduced with one (WA 1X) or two (WA 2X) hits of the w1.6W vector. As control, WASP expression in 129-wt lin⁻ cells is depicted. Numbers indicate VCN. (**b**) Engraftment of donor cells in sex-mismatched 129-*was*^{null} mice, as determined by Y chromosome-specific real-time PCR. For primary transplantations (p BM), dots represents measurements performed in total bone marrow cells, and horizontal bars depict the median value of the distribution. n = 4–6 mice per group. Arrows highlight mice chosen as donors for secondary transplants. For secondary transplantations, diamonds represent median ± interquartile range in total bone marrow cells (s BM, black) and spleen (s spl, white). wt GFP group: n = 10; WA 1X group: n = 9; WA 2X group: n = 9; *was*^{null} GFP group: n = 4. n.s. = not significant, Mann–Whitney test. (**c**) VCN in hematopoietic cells of mice recipient of either primary or secondary transplantation. VCN was determined by real-time PCR and normalized for the percentage of engraftment. For primary transplantations (p BM), dots represent measurements performed in total bone marrow cells, and horizontal bars depict the median value of the distribution. n = 4–5 mice per group. Arrows highlight mice chosen as donors for secondary transplants. For secondary transplantations, diamonds represent median ± interquartile range in total bone marrow cells (s BM, black) and spleen (s spl, white). wt GFP group: n = 10; WA 1X group: n = 9; WA 2X group: n = 8; *was*^{null} GFP group: n = 4. *P < 0.05, n.s. = not significant, Mann–Whitney test. (**d**) Survival curves of mice receiving secondary bone marrow transplantation. For wt GFP (filled squares), n = 11; for WA 1X (filled triangles), n = 16; for WA 2X (filled circles), n = 16; for *was*^{null} GFP (filled inverted triangle), n = 11. P < 0.05 for wt GFP group as compared to all the other groups, log-rank test. BM, bone marrow; VCN, vector copy number.

Table 2 Characteristics of hematopoietic tumors

Experiment	Mouse ID	Group	Histo-pathology	% Donor[a]	VCN[b]
12-month follow-up	645.6	Low MOI	Thymic lymphoma	2.5	0.04
12-month follow-up	648.4	Low MOI	Thymic lymphoma	2.3	0.1
12-month follow-up	663.1	Low MOI	Mesenteric LN lymphoma	1.1	0.1
12-month follow-up	664.3	Low MOI	Thymic lymphoma	0.7	0.03
12-month follow-up	611.2	lin$^-$ *was*^{null}	Thymic lymphoma	0.5	0.03
12-month follow-up	661.2	lin$^-$ *was*^{null}	Thymic lymphoma	1.8	ND
Secondary transplants	06-091-S20	WA 1X	Thymic lymphoblastic T-cell lymphoma	0.01	0.002
Secondary transplants	06-091-S25	WA 2X	Thymic early stage T-cell lymphoma	1	0.03
Secondary transplants	06-091-S24	WA 2X	Thymic lymphoblastic lymphoma	0	0

Abbreviations: LV, lentiviral vector; ND, not done; VCN, vector copy number.
[a]Percentage of donor cells as determined by Y-chromosome specific real-time PCR. [b]VCN per diploid genome as determined by LV specific real-time PCR. VCN was not normalized for the percentage of donor cells within the tumor.

and immunophenotyping of the BM and spleen (data not shown). Despite that the lifespan of secondary recipients of the *was*^{null} GFP, WA 1X, and WA 2X groups was lower than that of wt GFP group, while survival of mice belonging to the WA 1X and WA 2X groups was comparable to that of the *was*^{null} GFP group (**Figure 6d**). Evaluation of tumor occurrence could be performed in 7/11 mice in the *was*^{null} GFP group (64%), in 12/16 mice belonging to the WA 1X group (75%), in 13/16 mice in the WA 2X group (81%), and in 10/11 mice in the wt GFP group (91%). Based on macroscopic and histological examination, we observed no tumors in secondary recipients of wt GFP and *was*^{null} GFP cells (**Supplementary Table S3**). On the other hand, one secondary recipient belonging to the WA 1X group (8.3%) and two secondary recipients of the WA 2X group (15.4%) developed thymic lymphomas (**Table 2** and **Supplementary Table S3**). Molecular analysis indicated that all three lymphomas were of host origin and did not contain LV sequences (**Table 2**). In conclusion, serial transplantation experiments followed up for a cumulative period of 10 months did not reveal any toxicity related to the usage of the w1.6W LV.

DISCUSSION

In this study, we investigated the long-term efficacy and safety of WAS gene therapy using a *WAS* promoter/cDNA containing LV (w1.6W), compatible with future clinical application. *In vivo* WAS gene therapy studies performed up to now have addressed restoration of T-cell function in small cohorts of mice, with a maximal follow-up of 7 months.[26,31–33] By observing a large cohort (n = 68) of gene therapy treated *was*^{null} mice for 12 months, we could demonstrate the long-term stability of transgene expression, the occurrence of a selective advantage for WASP-expressing T and B lymphocytes, the persistence of T- and B-cell functional correction, and the improvement of B lymphocyte and platelet counts. Importantly, no donor-derived, LV-containing hematopoietic tumor developed in primary (n = 68) and secondary (n = 32) recipients of w1.6W-transduced BM cells, indicating that WAS gene therapy was very well tolerated.

An important rationale for the use of LVs is their high efficiency at transducing HSCs after a short *ex vivo* infection, which could favor the maintenance of stem cell properties.[23] Moreover, LVs are less genotoxic than LTR-active retroviral vectors,[24] and can harbor tissue specific internal promoters. Indeed, we and others have developed LVs expressing human WASP under the control of a 1.6 kb[34] or 0.5 kb[35] fragment of the proximal *WAS* promoter (w1.6W[13] and w0.5W[35] LVs, respectively). This strategy can allow the fine regulation of WASP expression in hematopoietic cells while avoiding toxicity due to off-target transgene expression.[13,36] Because WASP expression is regulated at both transcriptional and post-translational levels by highly specific interactions with transcription factors[25] and WIP,[37] respectively, it was crucial to compare WASP expression elicited by w0.5W and w1.6W LVs in human cells. Both w0.5W and w1.6W were comparable to various LVs containing constitutive promoters in eliciting WASP expression in T cells, B cells and CD34$^+$ HSCs derived from WAS patients, and in restoring the function of T cells and dendritic cells.[13,25] In addition, clonal analysis of LV-transduced, WAS patient-derived CD4$^+$ T-cell clones indicated that 1–2 copies of w1.6W and w0.5W LVs were sufficient to restore physiological WASP expression (**Supplementary Figure S1**). The w1.6W and the w0.5W LVs were also comparable in restoring T-cell activation and podosome formation in dendritic cells after gene therapy in *was*^{null} mice (**Supplementary Figures S2 and S3**). However, the w1.6W LV retained several transcription factor binding sites,[25] and was therefore selected for further preclinical development.

In accordance to our previous data,[26] the w1.6W LV-transduced murine lin$^-$ stem/progenitor cells at high efficiency after a short *ex vivo* infection. The stem cell properties of HSCs contained in the lin$^-$ population were likely well preserved, as suggested by durable engraftment of LV-transduced hematopoietic cells for at least 12 months in primary recipients, and for at least 6 months in secondary recipients (**Figures 1b,c** and **6b,c**). Importantly, a sublethal irradiation was enough to allow persistent multilineage engraftment of donor cells. This finding is in line with an anecdotal report about a WAS patient who successfully underwent matched

unrelated donor BMT after reduced intensity conditioning,[38] and suggests that reduced intensity conditioning could also be exploited as a less toxic preparatory regimen before administration of gene therapy to WAS patients.

One of the key issues for the success of gene therapy is whether WASP expression leads to a selective advantage of WASP$^+$ hematopoietic cells *in vivo*, causing the replacement of WASP-null cells in the case of mixed chimerism. In this work, we documented an *in vivo* enrichment for both T and B lymphocytes expressing WASP after gene therapy (**Figures 2b,c**). Our finding is in line with previous investigations demonstrating the enrichment of WASP$^+$ lymphocytes in *was* heterozygous female mice,[39] in *was*^{null} mice undergoing competitive BMT,[31] in WAS patients developing mixed chimerism after BMT,[40] in revertant WAS patients,[41] and in heterozygous female carriers.[42] The enrichment of WASP$^+$ lymphocytes could be due to the rescue of a developmental dysfunction in T and B cells,[39] and to a peripheral growth/survival advantage caused by preferential homing to secondary lymphoid organs,[7,11] efficient TCR-driven expansion of T cells,[12,13] and resistance to spontaneous apoptosis.[43] In spite of the strong enrichment of WASP-expressing lymphocytes, mixed chimerism could persist after gene therapy and could be a concern for the development of autoimmunity as recently reported in a cohort of WAS patients treated by allogeneic BMT.[19] However, the latter situation is complicated by allogeneic reactions between the host and donor cells, differently from gene therapy, which relies on autologous transplantation. In addition, we have evidences that gene therapy performed in 129-*was*^{null} mice prevents the onset of autoimmune colitis.[32,44]

Of note, low MOI WAS gene therapy was sufficient to correct T- and B-cell functional defects, suggesting that a clinical benefit could be obtained even in case of partial correction of WASP expression. This is in line with a retrospective study that correlated residual WASP expression in WAS patients with minimal immune dysfunction, lower clinical score, and extended lifespan.[45] Undoubtedly, highly efficient gene therapy, represented in this study by the high MOI group, broadens the number of corrected defects. Indeed, B cell and platelet counts in the high MOI group increased to levels reached by *was*^{null} mice transplanted with lin$^-$ wt cells (**Table 1**). Accordingly, high doses of WASP-encoding LVs were necessary to obtain functional correction of dendritic cells after WAS gene therapy,[32,46] while low LV doses were ineffective.[32] This defect could also be alleviated by high MOI gene therapy mediated by the w1.6W vector (**Supplementary Figure S3**). The average VCN in the high MOI gene therapy group was 2 in the BM and 4 in T cells, which might be considered high if it is directly translated to patients. However, the w1.6W was designed for optimal performance in human cells, where we expect to obtain physiological WASP levels with VCN 1–2 (refs. 13,25 and **Supplementary Figure S1**). Alternatively, sequences of the *WAS* distal promoter might be inserted to further improve the performance of the w1.6W LV, because this was recently reported to improve the performance of the shorter w0.5 promoter.[47]

Importantly, we found substantial evidence supporting the safety of WAS gene therapy mediated by the w1.6W LV. Indeed, 12-month survival of primary grafted mice of the low and high MOI gene therapy groups was comparable to that of mice belonging to lin$^-$ *was*^{null} and lin$^-$ wt control groups (**Figure 5a**). We observed a similar incidence of tumors (that were mainly of nonhematopoietic origin) in gene therapy treated mice as compared to controls (**Figure 5b**). Occurrence of host-derived hematopoietic tumors is a well-known confounding effect in the evaluation of preclinical models of gene therapy.[48] Therefore, molecular analysis is essential to determine the relationship between vector transduction and transgene expression with oncogenesis. In our study, lymphomas arose in four mice (8.7%) of the low MOI gene therapy group and in two mice (6.3%) of the lin$^-$ *was*^{null} group (**Figure 5b** and **Supplementary Table S1**), but were all of host origin and did not bear LV integrations, as confirmed by molecular analysis (**Table 2**). Mice were followed for 12 months upon transplantation, a time window shorter as compared to a clinical follow-up but long enough to observe adverse events in murine models of gene therapy for common-γ chain or CD40L deficiencies.[27,28] In contrast, in our study none (0/68) of the primary grafted animals developed donor-derived, LV-transduced hematopoietic tumors up to 12 months after gene therapy. In some experimental settings, oncogenicity of gene transfer could be evidenced by secondary BMT.[29] However, in our secondary transplantation experiments spanning a period of 10 months, transduction of HSCs with the w1.6W LV did not cause tumorigenesis. Indeed, survival of *was*^{null} mice that received secondary transplantation of w1.6W-trasduced BM cells was comparable to that of *was*^{null} mice receiving secondary transplantation of GFP-transduced BM cells (**Figure 6d**). Although we documented the occurrence of 3 lymphomas out of 25 evaluable secondary recipients of w1.6W-transduced BM (12%, see **Supplementary Table S3**), they were of host origin and did not bear LV integrations (**Table 2**). Noteworthy, in our study we evaluated a total amount of integration events, calculated as being about 63×10^6, comparable to a single patient's dose (estimated as 75×10^6 events for a patient of 15 kg receiving 5×10^6 CD34$^+$ cells/kg with an average VCN = 1).

In addition to the experimental data we provide in this work, other considerations account for the safety of a WAS gene therapy approach using LVs. Indeed, *WAS* is not classified as a cancer related gene (negative search in the Cancer Gene Expression Database, http://lifesciencedb.jp/cged/, and in the Retrovirus Tagged Cancer Gene Database, http://rtcgd.abcc.ncifcrf.gov/, accessed November 2008), and WASP expression levels are controlled by post-transcriptional mechanisms.[49] Moreover, LVs offer a better safety profile as compared to retroviral vectors.[24] Taken together, these data strongly account for the safety of WAS gene therapy mediated by the w1.6W LV. The demonstration of long-term efficacy and safety of WAS gene therapy using the w1.6W LV presented in this work is critically contributing to the implementation of a multicentre WAS gene therapy clinical trial.

MATERIALS AND METHODS

Mice. C57BL/6 *was*^{null} mice (BL6-*was*^{null})[15] were crossed with C57BL/6-CD45.1 mice to obtain the BL6-*was*^{null}-CD45.1 strain at the San Raffaele Scientific Institute animal facility in Milan, Italy. This strain is characterized by long lifespan after sublethal irradiation and by marked thrombocytopenia, and therefore it was chosen for long-term observation studies and for the evaluation of platelet counts after gene therapy. A distinct *was*^{null} mouse model under the 129sv background (129-*was*^{null}) was kindly provided by S.B. Snapper,[14] and housed in Généthon (Evry, France).

This strain has a shorter lifespan due to colitis exacerbation after irradiation, and was therefore used for serial grafts. Control wt mice were purchased from Charles River Laboratories (Calco, Italy) or Iffa Credo (L'Arbesle, France). All mice used in this study were housed in specific pathogen-free conditions and subjected to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute or by Généthon.

LVs. The w1.6W vector is a self-inactivating LV constructed on the pRRL backbone and encoding for WASP as the only transgene.[13,25,26] The transcription of human *WAS* cDNA is controlled by a 1.6 kb sequence of the autologous proximal *WAS* promoter.[34] A Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) sequence was included in the vector, aiming at increasing transgene expression by transcript stabilization. The w1.6W vector is VSVg pseudotyped. The final configuration of the w1.6W vector, that will be used for large scale good manufacturing procedures production oriented to clinical application, was obtained by swapping it on the pCCL backbone, and substituting the wild-type WPRE with a mutated WPRE sequence (WPREmut6) lacking the transcription of a fragment of the potentially oncogenic woodchuck hepatitis virus-X protein.[50] The performance of the w1.6W vector was unaltered by those changes (S. Charrier and A. Galy, unpublished results), and both were used in this study. A GFP-encoding LV (pGFP) was used as control in some experiments. Two additional pRRL vectors encoding WASP from the w0.5 promoter or from the PGK promoter were used in supplemental studies and have been described earlier.[25] For this study, nongood manufacturing procedures small scale LV productions were made by co-transfection of transgene-encoding transfer plasmids with packaging plasmids encoding *gag/pol*, *VSV-G env*, and *rev* into 293T cells. Harvested virus particles were concentrated by ultracentrifugation. Virus p24 concentration was measured by enzyme-linked immunosorbent assay. Viral titer was determined by infection of HCT116 cells with serially diluted virus preparations followed by proviral sequence detection, as previously described.[32]

Immunophenotype and western blot analysis. The following mAbs were used for surface staining: anti-CD3 (17A2), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b/Mac1 (M1/70), anti-CD45R/B220 (RA3-6B2), anti-CD45.2 (104), anti-CD90.2 (53-2.1), all from BD Pharmingen (San Diego, CA). Intracytoplasmic detection of human and murine WASP was performed using the anti-WASP antibody 503 (a kind gift of H. Ochs and L.D. Notarangelo) after fixation and permeabilization of the cells using the Cytofix/Cytoperm kit (BD Pharmingen). Western blot analysis was performed as previously described.[32] Membranes were probed with the B9 or H250 anti-WASP Abs (crossreactive between human and murine WASP; Santa Cruz Technologies, Santa Cruz, CA), the anti-β-actin mAbs AC-15 (Sigma, St Louis, MO), and the MAB374 anti-GAPDH mAbs (Chemicon, Temecula, CA).

Real-time PCR analysis. Genomic DNA was extracted from hematopoietic cells using the QIAmp DNA Blood mini-kit (Qiagen, Hilden, Germany). DNA extraction from paraffin-embedded samples was performed either using the QIAmp DNA FFPE Tissue Kit (Qiagen) or the Agilent protocol available online (http://www.chem.agilent.com/cag/prod/dn/G4410-90020_CGH_Protocol_FFPE1_0.pdf). LV copy number (VCN) per diploid genome, and percentage of cells carrying the Y chromosome, were assessed by absolute quantification using the primers listed in **Supplementary Table S4.** Measurement of the human WAS mRNA relative to that of the murine TF2D mRNA was performed as previously reported.[32]

Long-term follow-up experiments. Long-term follow-up experiments were performed using BL6-*was*^{null} and wt mice. Lineage marker–depleted BM (lin$^-$) cells were purified and transduced using a previously reported protocol.[26] Transduction was performed by culturing 1×10^6 lin$^-$ cells in the presence of $1–2 \times 10^7$ or 2×10^8 infectious viral genomes (ig) per ml of culture (MOI = 10–20 and 200, respectively) for 12 hours. Lin$^-$ cells ($0.25–1 \times 10^6$ cells/mouse) were transferred intravenously into sublethally irradiated (700 rad) 6- to 8-week-old *was*^{null} sex- or CD45 allele-mismatched recipient mice. Some transduced lin$^-$ cells were grown *in vitro* for more than 7 days to analyze transduction efficiency. Twelve months after transplantation, splenic T cells were isolated by means of immunomagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) and stimulated for 48 hours with plate-bound anti-CD3 mAbs (clone 17A2 from BD Pharmingen, 2 μg/ml) as previously described.[26] Proliferation was measured by ^{3}H-thymidine pulsing and liquid scintillation counting. For each sample, the stimulation index (*i.e.*, the ratio between counts per minute of stimulated versus nonstimulated cells) was calculated. Concentration of IL-2, interferon-γ, tumor necrosis factor-α, IL-4, and IL-10 was assessed by BioPlex technique (Bio-Rad Laboratories, Hercules, CA) in conditioned supernatants. To assess B-cell migration in response to CXCL13 (Peprotech, Rocky Hill, NJ), CD45R/B220$^+$ splenic B cells were selected by means of immunomagnetic beads (Miltenyi) and seeded in duplicate on the upper well of a 5-μm transwell (Corning Costar, Corning, NY) at the concentration of $1–2 \times 10^6$/ml in 100 μl of medium. In the bottom well, 600 μl of medium supplemented with 1 μg/ml of CXCL13 were placed. Cells were incubated for 6 h at 37 °C, and cells migrated to the lower well were counted. Percentage of migration was calculated as compared to the input cell number.

Secondary transplantation experiments. Secondary transplantation experiments were performed using 129-*was*^{null} or wt mice. Lin$^-$ cells were purified and cultured as previously reported,[32] with minor modifications. After 1 day of cytokine prestimulation, male *was*^{null} lin$^-$ cells were infected with the w1.6W vector at the concentration of 1×10^8 ig/ml (MOI = 100) for 6 hours, and then washed. Part of the cells received a second vector hit (MOI = 100) administered overnight, and then washed. As control, male *was*^{null} or wt lin$^-$ cells were transduced once with the pGFP vector (1×10^8 ig/ml, MOI = 100). One day after infection, lin$^-$ cells (0.5×10^6 cells/mouse) were transferred intravenously into *was*^{null} sex-mismatched female mice, which had been lethally irradiated (950 rad) 2–3 hours prior to transplantation. Some of the transduced lin$^-$ cells were cultured *in vitro* for 7 days for analysis of transduction efficiency. Four months after primary graft, mice with the highest VCN or Y chimerism in peripheral blood were chosen as BM donors for secondary transplants. Total BM cells were collected and transplanted (2×10^6 cells/mouse) into lethally irradiated (950 rad) female secondary *was*^{null} recipients. Mice were followed up for 6 months.

Animal follow-up, hemogram analysis, and histopathologic evaluation. Animals were weekly evaluated to monitor their general health status. At the end of programmed follow-up, or in case of compromised health status, mice underwent complete blood cell count and were euthanized to perform full macroscopic and histopathologic analysis of hematopoietic and nonhematopoietic tissues. For hemogram analysis in the long-term follow-up experiments, blood samples were collected in 4.5% EDTA and analyzed with a KX21N counter (Sysmex, Kobe, Japan). Absolute numbers of T cells, B cells, monocytes, and granulocytes were determined as reported previously.[26] For secondary transplants, blood was collected in 3.8% citrate solution, and analyzed using an MS9.3 counter (Schloessing Melet, Cergy-Pontoise, France). For histopathologic evaluation, organs (thymus, colon, small intestine, spleen, lymph nodes, liver, kidney, and bone) of all killed mice were formalin-fixed and paraffin-embedded. Tissue sections (4 μm thick) were stained with hematoxylin and eosin and evaluated by certified pathologists with experience in rodent tissue analysis (M.P., F.S., C.D. in Milan and by Biodoxis, an independent pathologist subcontractor, in Evry). Tumor samples were subjected to qPCR analysis, to assess their donor or host origin and the presence of integrated LV sequences.

Statistical analysis. In case of Gaussian distribution of the data (checked by Kolmogorov–Smirnov test), experimental groups were compared with a two-tailed Student *t*-test. Otherwise, a two-tailed Mann–Whitney test was used. P values <0.05 were considered significant.

SUPPLEMENTARY MATERIAL

Table S1. Lifespan, details of necropsy and histopathologic analysis, and blood cell counts of all BL6-*was*^{null} mice followed up for 12 months after gene therapy.

Table S2. Lifespan, details of necropsy and histopathologic analysis, and blood cell counts of all primary grafted 129-*was*^{null} mice.

Table S3. Lifespan, details of necropsy and histopathologic analysis, and blood cell counts of all secondary grafted 129-*was*^{null} mice.

Table S4. The list of primers for real-time PCR used in this study.

Figure S1. The analysis of WASP expression and vector copy number in WAS patient's CD4$^+$ T cell clones, after transduction with three WAS-encoding vectors (in which WASP is controlled by the PGK, the w1.6 or the w0.5 promoter).

Figure S2. The comparison between three WAS-encoding vectors (in which WASP is controlled by the PGK, the w1.6 or the w0.5 promoter) in the restoration of T cell activation after gene therapy.

Figure S3. The comparison between three WAS-encoding vectors (in which WASP is controlled by the PGK, the w1.6 or the w0.5 promoter) in the restoration of podosome formation by bone-marrow derived DCs.

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