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ARTICLE

Lentiviral Vector-Mediated Gene Transfer in T Cells from Wiskott–Aldrich Syndrome Patients Leads to Functional Correction

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Wiskott–Aldrich syndrome (WAS) is an X-linked primary immunodeficiency with a median survival below the age of 20 due to infections, severe hemorrhage, and lymphomas. Transplantation of hematopoietic stem cells from HLA-identical sibling donors is a resolutive treatment, but is available for a minority of patients. Transplantation of genetically corrected autologous hematopoietic stem cells or T cells could represent an alternative treatment applicable to all patients. We investigated whether *WAS* gene transfer with MMLV-based oncoretroviral and HIV-based lentiviral vectors could restore normal functions of patients' T cells. T cells transduced either with lentiviral vectors expressing the WAS protein (WASP) from the ubiquitous PGK promoter or the tissue-specific WASP promoter or with an oncoretroviral vector expressing WASP from the LTR, reached normal levels of WASP with correction of functional defects, including proliferation, IL-2 production, and lipid raft upregulation. Lentiviral vectors transduced T cells from WAS patients at higher rates, compared to oncoretroviral vectors, and efficiently transduced both activated and naïve WAS T cells. Furthermore, a selective growth advantage of T cells corrected with the lentiviral vectors was demonstrated. The observation that lentiviral vector-mediated gene transfer results in correction of T cell defects *in vitro* supports their application for gene therapy in WAS patients.

Key Words: Primary immunodeficiency, T lymphocytes, Lentiviral vectors, Oncoretroviral vectors, Functional correction

INTRODUCTION

Wiskott–Aldrich syndrome (WAS) is a severe X-linked immunodeficiency characterized by recurrent infections, eczema, and thrombocytopenia [1]. WAS is caused by mutations in the gene encoding the WAS protein (WASP), which is expressed in hematopoietic cells and plays a key role in organizing the actin cytoskeleton [2–4]. The absence of WASP has been associated with impairments in several cellular functions, including motility [5–7], antigen uptake by endocytosis [8], adhesion [9], and activation [10,11]. The molecular mechanism of action of WASP has been characterized in T cells, which need WASP expression for optimal activation through the T cell receptor (TCR) [10–13]. Recently, we demonstrated that WASP sets the threshold for TCR-driven activation by regulating the dynamics of lipid raft membrane microdomains during immunological synapse formation [14].

Currently, the only resolutive therapeutic option for WAS is hematopoietic stem cell transplantation (HSC-T) [15,16]. However, when HLA-identical siblings are not available and transplantation is performed with HSC from HLA-matched or -haploidentical donors, limited success has been reported in comparison to other forms of congenital immunodeficiencies, due to graft failure, graft-versus-host-disease, severe infections, or EBV-induced B lymphocyte proliferative disorders. Over the

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past decade, earlier diagnosis, successful HSC-T, and careful supportive therapy has extended significantly the life expectancy of WAS patients. However, the prolonged life span of patients not cured by HSC-T has been associated with an increased incidence of lymphomas and autoimmune disorders [17,18]. The development of gene therapy approaches based on the correction of autologous hematopoietic cells has thus become a priority goal for the cure of this disease because it could overcome the current limitations and morbidity of allogeneic HSC-T and could be applicable to all WAS patients. The gene therapy trials on SCID-X1 and ADA-SCID patients based on oncoretroviral vector-mediated gene transfer into peripheral blood T cells or HSC have shown that the selective advantage of corrected cells is a key factor for successful immune reconstitution [19–21]. There is increasing evidence that a selective advantage for WASP-expressing T cells over WASP-null cells exists *in vivo*. Spontaneous *in vivo* reversion of WASP mutation leading to T cell functional correction, and in some cases to progressive clinical improvement, has been reported in a number of WAS patients [22–25]. In addition, when HSC-T results in partial chimerism with engraftment of only T cells, the immune defect is corrected, indicating that the absence of WASP in these cells is the primary factor responsible for the development of WAS immunodeficiency [26]. Thus, correction of the T cell defect is a prerequisite for successful gene therapy targeting either peripheral lymphocytes or HSC from WAS patients.

We and others have previously reported that gene transfer into WAS patients' T cells with oncoretroviral vectors encoding WASP under the control of LTR or internal promoters resulted in low gene transfer efficiencies but was able to correct at least partially the proliferative defects of transduced cells [14,27,28]. In addition, transplantation of oncoretroviral vector-transduced murine WASP-KO HSC into immunodeficient recipients or autologous WASP-KO animals resulted in the differentiation of T cells with a partial correction of their proliferative response to TCR stimulation and of their specific response to influenza virus [29,30]. Complete phenotype correction of WAS T cells might not have been reached with oncoretroviral vectors because the levels of vector-derived WASP expression were lower than normal. Indeed, since WASP sets the threshold for TCR/CD3 stimulation [14], it can be predicted that complete functional restoration will require a level of WASP expression comparable to normal. In addition, the retroviral vectors developed so far would not be compatible with a use in human trials since they encoded either murine WASP or reporter genes such as GFP. The design of efficient clinically applicable vectors is thus required. Self-inactivating HIV-based lentiviral vectors infect hematopoietic cells more efficiently than oncoretroviral vectors, resulting in overall higher levels of transgene expression and possibly ensuring more efficient long-term correction of progenitor cells. Furthermore, the design of lentiviral vectors with the WASP autologous promoter [31,32], upstream of the WASP cDNA, would allow a physiological regulation of WASP, as demonstrated for other promoter/transgene constructs [33,34].

In this study, we investigated whether the use of advanced-design lentiviral vectors, encoding WASP under a strong ubiquitously-active promoter or the autologous WASP promoter, leads to higher gene transfer efficiency and complete correction *in vitro* of WAS T cells, compared to oncoretroviral vectors.

RESULTS

Gene Transfer with WASP-Encoding Oncoretroviral Vectors

We constructed two MMLV-based oncoretroviral vectors containing the WASP cDNA under the control of either

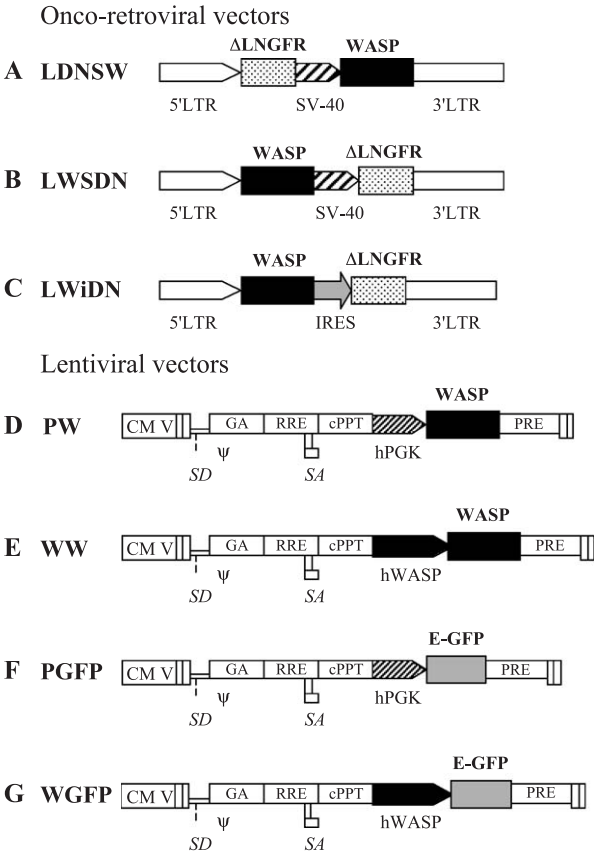

FIG. 1. Schematic representation of the oncoretroviral and lentiviral vectors. (A–C) MMLV-based oncoretroviral vectors encoding WASP and ΔLNGFR with WASP under the control of the SV40 internal promoter or the MMLV LTR. (D and E) HIV-1-based lentiviral vectors encoding WASP under the ubiquitous PGK promoter or the autologous WASP promoter. (F and G) HIV-1-based lentiviral vectors encoding E-GFP under the PGK promoter or the WASP promoter. Note that the lentiviral vectors are self-inactivating constructs and that the CMV promoter is present only in the producer cells.

the SV40 internal promoter or the MMLV LTR (Figs. 1A and 1B). We established stable Am12-based producer cell lines and subcloned them for the selection of clones with high transduction capacity. Only 5 of 50 clones tested produced viral vectors with detectable capacity to transduce preactivated PBMC from normal donors (data not shown). We analyzed these 5 clones and 5 additional ones for WASP expression by Western blot (Fig. 2A). WASP expression levels were highly variable, ranging from low to high (comparable or superior to the levels measured in normal T cells). Surprisingly, the 3 clones expressing the lowest levels of WASP were the ones displaying the best production of infective particles, as measured by the efficiency of transduction of preactivated WAS patients' T cells and the titer on NIH3T3 cells (Figs. 2B and 2C). In addition, only the clones with the lowest WASP expression secreted detectable envelope protein (Fig. 2D), whereas both clones with low and high WASP expression secreted normal amounts of the gag–pol protein (Fig. 2E). These data suggest that stable expression of WASP in the Am12 producer cells could interfere with the process of viral vector assembly and budding. Alternatively, a rearrangement in the *env* gene in some of the clones may be responsible for the low envelope protein detected. We selected the two oncoretroviral vectors derived from the bulk producer cells to transduce WAS patients' T cells. Transduction levels were low, ranging between 2 and 10%. Thus, we used two successive rounds of immunoselection to obtain purified transduced cell populations (Fig. 3A). As expected from the respective strengths of the LTR and SV40 promoters, the LDNSW vector led to higher levels of ΔLNGFR expression (measured by mean fluorescence intensity (MFI) after immunostaining) than the LWSDN vector. To improve gene transfer efficiency and to circumvent the possible toxic effect of WASP on the stable packaging cells, we developed a transient production system, based on transient transfection into 293T cells of the LWiDN construct (Fig. 1C). Using this protocol, we obtained higher titers of WASP-encoding viruses (4–6 × 10^6 CFU/ml), compared to the stable packaging system, with higher transduction efficiency ranging from 10 to 20%. Thus, only one round of immunoselection was required to obtain purified transduced cells (Fig. 3B).

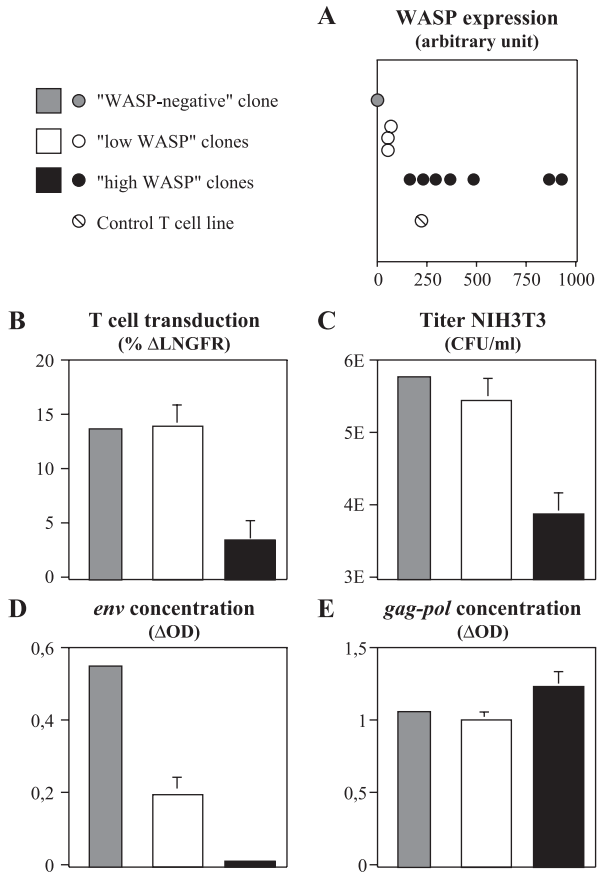

FIG. 2. Characteristics of WASP-encoding retroviruses produced from stable Am12 packaging cell clones. (A) Distribution of WASP expression in 10 packaging cell clones stably transduced with WASP-encoding constructs. The clones were classified as expressing either low or high WASP levels. As controls, WASP expression in an untransformed T cell line and in a packaging cell clone containing a ΔLNGFR-encoding construct are shown. The levels of WASP expression were measured by densitometric analysis of a Western blot. (B) Percentage of ΔLNGFR-positive WAS T cells after incubation with WASP-encoding MMLV retroviruses produced by Am12 packaging clones expressing WASP at either low or high levels, as indicated. (C) Titer of retroviruses produced by the Am12 packaging clones tested on NIH3T3 cell line (3E = 1×10^3). (D and E) Concentration (expressed as OD) of the envelope and gag–pol proteins, respectively, measured by ELISA in the supernatants of the Am12 packaging clones. For all the parameters measured an Am12 clone expressing ΔLNGFR under the control of the SV40 promoter was used as a control (gray dot and bars).

Efficient WAS T Cell Transduction with Lentiviral Vectors

We constructed third-generation lentiviral vectors with the WASP cDNA under the control of either the human PGK ubiquitous promoter (PW) or a 1.6-kb genome fragment from the human WASP promoter (WW) (Figs. 1D and 1E). The concentrations of lentiviral vectors produced by transient transfection of 293T cells were in the range observed with other transgenes (60–80 μg/ml p24). Interestingly, to retain a good infectivity, WASP-encoding viral vectors had to be collected within 40 h after the transfection, since later collection resulted in the recovery of viral vectors with poor infectivity on HeLa cells, while the p24 value was comparable at both early and late time points (data not shown). These data suggest that, as for oncoretroviral vectors, WASP expression might exert a toxic effect on lentiviral vector production. We performed transduction of patients' T cells with lentiviral vectors under the same culture conditions used for the oncoretroviral vectors. We first optimized the transduction protocol with a control lentiviral vector

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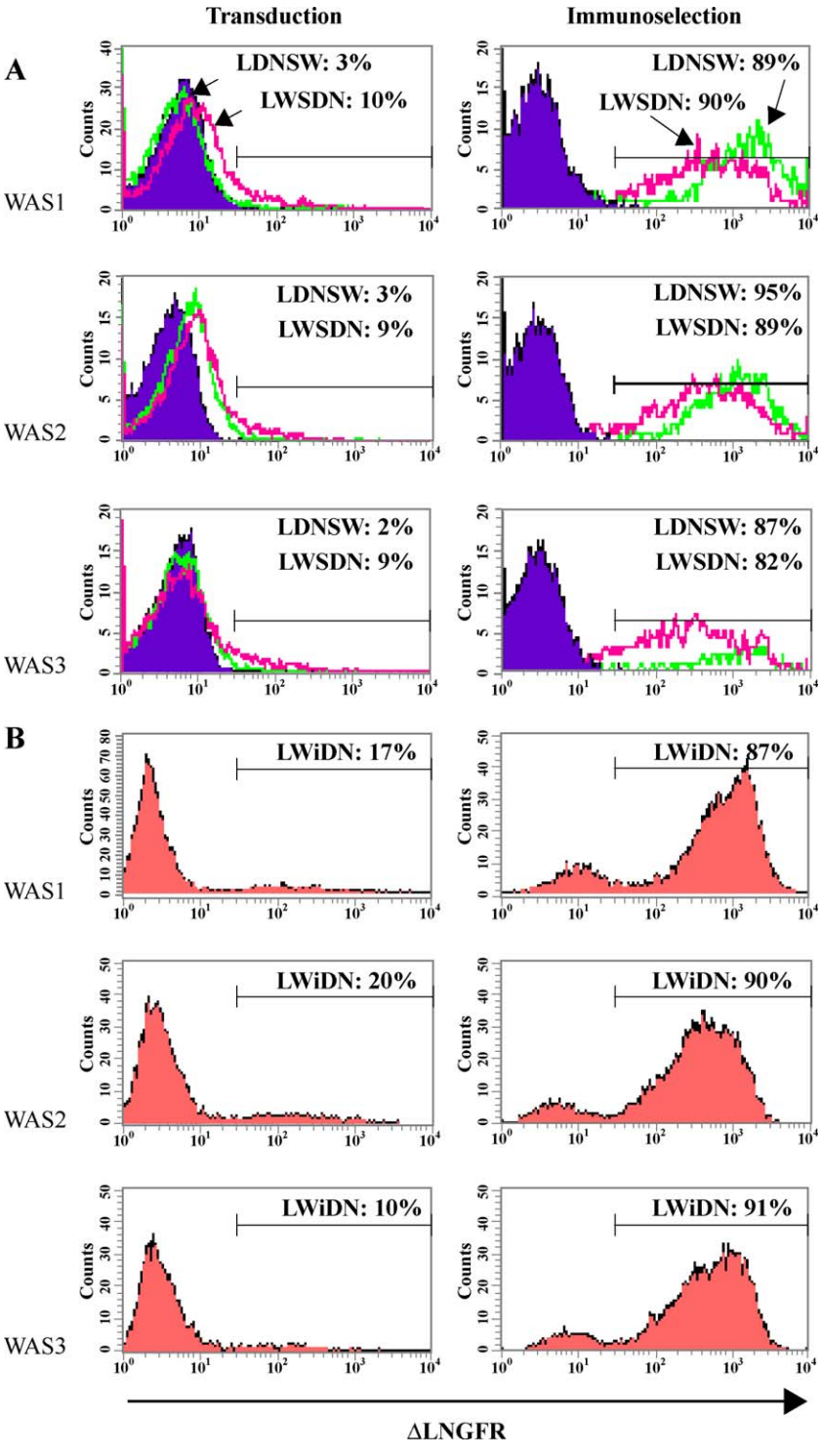

FIG. 3. Reporter gene expression after transduction of WAS patients' T cell lines and PBMC with MMLV oncoretroviral vectors and lentiviral vectors. (A) FACS analysis of ΔLNGFR expression in WAS T cells transduced with LDNSW or LWSDN oncoretroviral vectors produced from bulk Am12 packaging lines, either directly after transduction or after two rounds of immunoselection (one representative of five experiments). (B) FACS analysis of ΔLNGFR expression in WAS T cells transduced with LWiDN oncoretroviral vector directly after transduction and after one round of immunoselection (representative of two experiments). (C) FACS analysis of E-GFP expression in WAS (empty squares) and normal (filled circles) T cells 4 days after transduction with a PGFP lentiviral vector at the indicated virus concentrations (representative of two experiments). (D) FACS analysis of E-GFP and CD45RA in WAS1 patient's PBMC 10 days after transduction with PGFP or WGFP, as indicated. Transduction was performed either after 48 h TCR prestimulation ("TCR" : anti-CD3 and CD28 mAbs, plus IL-2 and IL-7) or after 96 h cytokine prestimulation ("cyto": low doses of IL-2 and IL-7) using in both cases a virus concentration of 15×10^6 TU/ml. Numbers in the quadrants indicate percentages of transduced naïve T cells (positive for both E-GFP and CD45RA). In one experiment, PGFP/cyto was compared to PGFP/TCR and in a second experiment, PGFP/cyto was compared to WGFP/cyto.

expressing E-GFP under the control of the PGK promoter (Fig. 1F). Transduction efficiency in WAS T cells reached up to 60–90% and was generally slightly higher than that of control T cells. A short incubation with the viral vector (4 h) was sufficient to reach high transduction. The plateau value was observed with a viral vector concentration of 8×10^6 transducing units (TU)/ml after the 4-h transduction and of 4×10^6 TU/ml after the 24-h

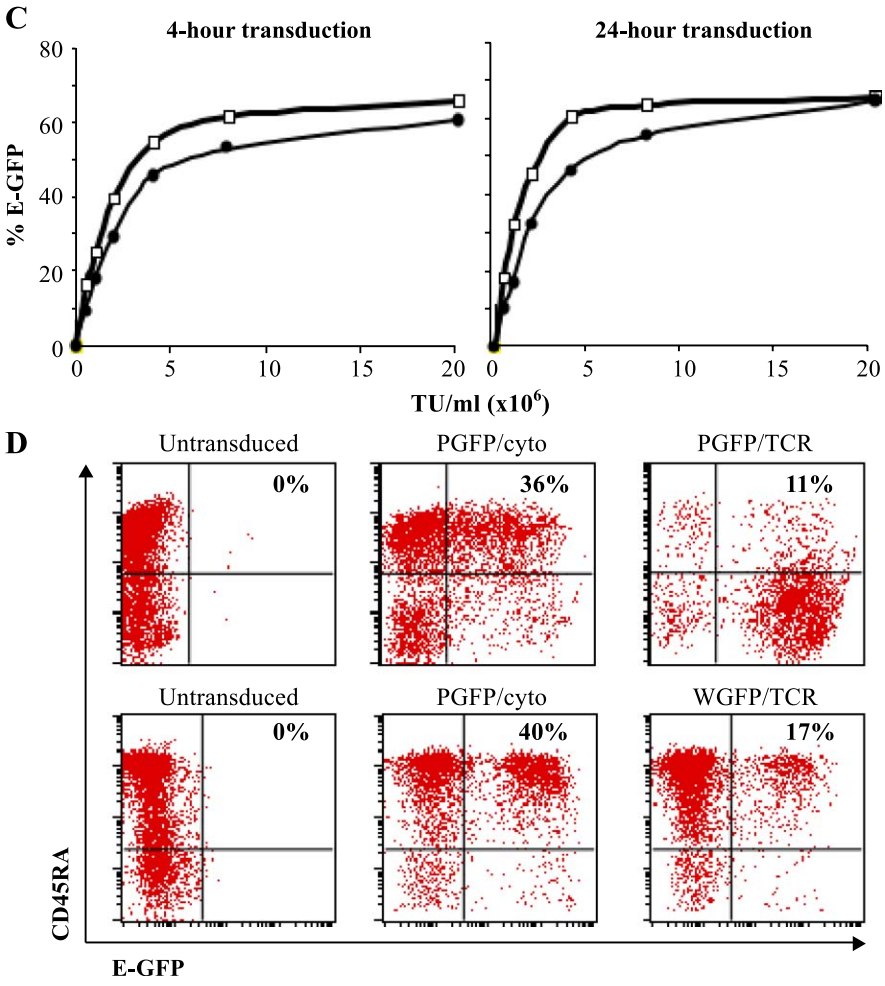

FIG. 3 (*continued*).

transduction (Fig. 3C). Since lentiviral vectors are able to infect nondividing cells, we tested the possibility of transducing WAS patients' PBMC in the absence of TCR/CD28 triggering, to favor the maintenance and the transduction of naïve T cells. Fig. 3D shows the FACS analysis of E-GFP and CD45RA expression in WAS1 patient's PBMC 10 days after one round of infection with either the PGFP or the WGFP lentiviral vector. When we prestimulated the PBMC by polyclonal activation with anti-CD3 and CD28 mAbs, plus IL-2 and IL-7, transduction efficiency with PGFP reached 87%. However, only a minority of those transduced cells preserved a naïve phenotype (11% GFP-positive/CD45RA-positive T cells). In contrast, after 4 days of prestimulation with low doses of IL-2 and IL-7, and infection with either the PGFP or the WGFP lentiviral vectors, most of the patient's T cells preserved a naïve phenotype. The overall transduction efficiency was lower after cytokine (18 to 44%) than after TCR stimulation, but the percentage of transduced naïve WAS T cells reached higher levels (17 to 40%).

Preferential Expression in Hematopoietic Cells with the WASP promoter

To test the strength and the tissue specificity of the 1.6-kb genome fragment from the human WASP promoter that was cloned in the backbone of our lentiviral vectors, we constructed and compared the E-GFP-encoding vectors PGFP and WGFP (Figs. 1F and 1G) for their ability to drive E-GFP expression in hematopoietic versus nonhematopoietic cell lines. We performed transductions in parallel with both viruses at a dose of 0.1 ng p24 per 100,000 cells. We measured fluorescence intensity of E-GFP after 72 h by FACS in the fraction of positive cells. To normalize the intensity of E-GFP expression, we calculated the ratio between E-GFP MFI obtained after transduction with WGFP and E-GFP MFI obtained after transduction with PGFP for each cell line. Overall, the

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WGFP vector led to lower E-GFP expression levels than the PGFP vector since the ratios were all below 1 (Fig. 4). However, transgene expression from the WASP promoter in the context of the lentiviral vector was stronger in the hematopoietic cell lines (Jurkat, JY, HEL, U937) compared to the nonhematopoietic cell lines (HeLa, NIH3T3, 293T). This observation was also confirmed at other doses of viruses tested. The preferential expression in hematopoietic cells was more evident for the T cell line (Jurkat) and the erythroid cell line (HEL) than for the macrophage cell line (U937) and the B cell line (JY).

Levels of WASP Expression in WAS Patients' T Cells after Gene Transfer

We first assessed levels of vector-derived WASP expression in transduced patients' T cells by Western blot analysis. As shown in Fig. 5A, WASP was not detected in control vector-transduced WAS T cells. After transduction with LWSDN or LDNSW oncoretroviral vectors and two rounds of immunoselection for ΔLNGFR expression, T cells from the three WAS patients expressed WASP at the expected molecular weight. WASP expression levels were slightly higher after transduction with the LWSDN vector, compared to the LDNSW vector. However, these levels were significantly lower than those of normal T cells (Fig. 5A). After transduction with the LWiDN oncoretroviral vector and one round of immunoselection for ΔLNGFR expression, T cells from the three patients expressed high levels of WASP, which were comparable to those of normal control T cells (Fig. 5B). High levels of WASP were also expressed after transduction with the PW or WW lentiviral vectors, despite the fact that transduced cells were not enriched by immunoselection (Fig. 5C). At our standard concentration of lentiviral vectors (100 ng p24/1 $\times$ 10^5 T cells in a volume of 0.5 ml), WASP expression in transduced WAS1 T cells was slightly lower compared to a normal T cell line. However, when the vector concentration was increased (500 ng p24/1 $\times$ 10^5 T cells in a volume of 0.5 ml), WASP expression reached levels comparable to normal. Importantly, the WW vector containing a fragment from the WASP promoter led to a very similar restoration of WASP expression, compared to the PW vector containing the PGK promoter (Fig. 5C). Comparable results were obtained in all three patients' T cell lines (data not shown). Intracytoplasmic WASP staining and FACS analysis showed that T cells transduced with the LWSDN retrovirus and immunoselected for ΔLNGFR expression represented a fairly homogeneous population, which expressed WASP at intermediate intensity compared to untransduced WASP-deficient cells and normal control cells (Fig. 5D). In contrast, T cells transduced with the transiently produced LWiDN oncoretroviral vector and purified based on ΔLNGFR surface expression, expressed levels of WASP similar to those of normal control T cells (Fig. 5E). Cells transduced with both the PGK promoter- and the WASP promoter-containing lentiviral vectors expressed from intermediate to high levels of WASP. The initial percentage of WASP-positive cells ranged from 24 to 98% (measured in transduced T cells from the three WAS patients in five independent experiments) and was found to be very comparable when the same patient's T cells were transduced with either lentiviral vectors (Fig. 5F). In parallel to the analysis of WASP expression in the transduced WAS T cells, we determined vector copy number by quantitative PCR. Using the vector dose of 100 ng p24/1 $\times$ 10^5 T cells in a volume of 0.5 ml, T cells contained 1.7 to 3.0 copies of the PW vector per cell, while they contained 0.7 to 1.3 copies of the WW vector per cell. Thus, the WASP promoter fragment appeared to be as strong as the PGK promoter in the context of our third-generation lentiviral vectors. In addition, our results indicate that low integrated copy numbers of both lentiviral vectors were sufficient to restore normal levels of WASP expression in WAS patients' T cells. Importantly, when we cultured the WAS T cell lines transduced at the lowest efficiencies for several weeks, a significant increase in the fraction of WASP-positive cells was observed over time (Fig. 6). In the T cell lines transduced with the PGK promoter-containing vector, the proportion of WASP-positive cells increased from 37% at week 8 to 59% at week 14 ($P < 0.01$, t test) and then stabilized at this value. The progressive increase in WASP-positive cells was significantly more pronounced for the T cell lines transduced with the WASP promoter-containing vector, since they were 42% at week 8 and

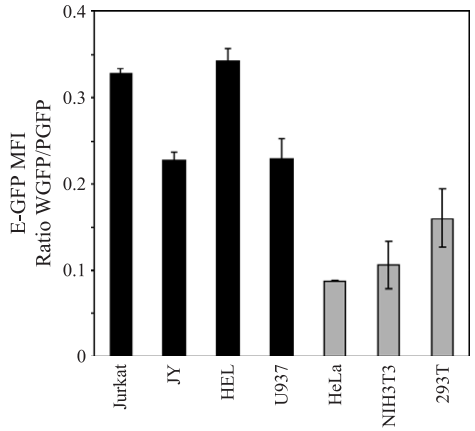

FIG. 4. Relative strengths of the PGK and WASP promoters in hematopoietic versus nonhematopoietic cells. Mean fluorescence intensity of E-GFP was measured by FACS 72 h after transduction of the indicated cell lines with the PGFP and WGFP lentiviral vectors used at the same concentration (0.1 ng p24/1 $\times$ 10^5 cells/0.5 ml). To normalize the intensity of E-GFP expression, the ratio between E-GFP MFI obtained after transduction with WGFP and E-GFP MFI obtained after transduction with PGFP is shown. Hematopoietic cell lines (Jurkat, JY, HEL, U937) are indicated in black, while nonhematopoietic cell lines (HeLa, NIH3T3, 293T) are indicated in gray. Each bar represents the mean $\pm$ SD of the WGFP/PGFP ratios calculated from transductions performed in triplicate with the two vectors in parallel.

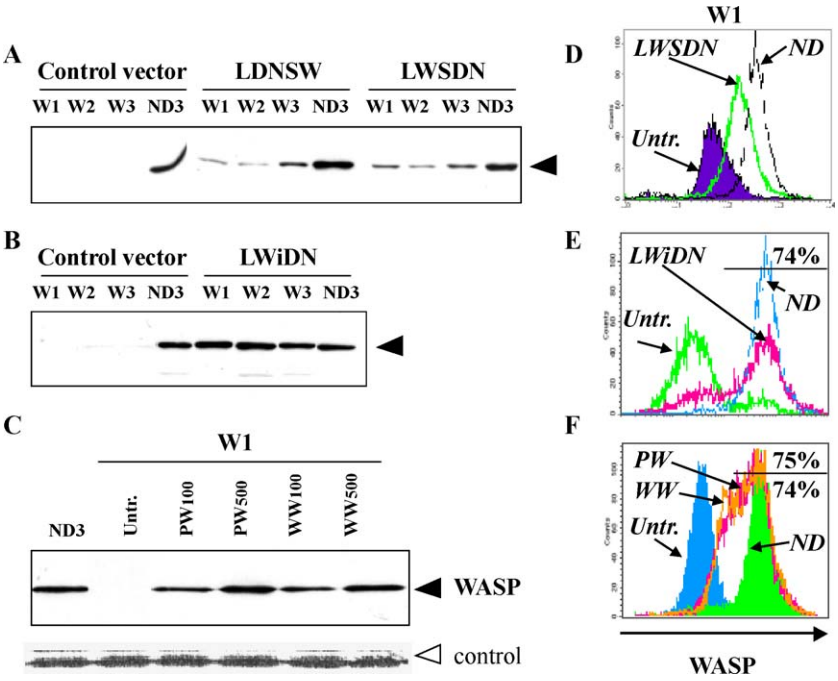

FIG. 5. Analysis of WASP expression after transduction with MMLV oncoretroviral vectors and lentiviral vectors by Western blot and by FACS. (A) Analysis of WASP expression by Western blot in lysates of T cells (W1, W2, W3 indicate T cells from the three WAS patients, and ND3 indicates T cells from a normal donor) after transduction with the stably produced oncoretroviral vectors (LDNSW, LWSDN, and SFCMM3 as control) and immunoselection. (B) Analysis of WASP expression by Western blot in lysates of T cells after transduction with the transiently produced oncoretroviral vectors LWiDN (and pRRV-IRES/ΔLNGFR as control) and immunoselection. (C) Analysis of WASP expression by Western blot in lysates of WAS1 T cells 20 days after transduction with the PW and WW lentiviral vectors at two different concentrations (100 and 500 ng p24). Staining of one portion of the blot with Ponceau red (indicated as control) shows comparable protein loading in each lane. (D) Analysis of WASP expression by intracytoplasmic flow cytometry of WAS1 T cells after transduction with the stably produced oncoretroviral vector LWSDN and immunoselection. Stainings of untransduced WAS1 T cells (Untr.) and normal donor T cells (ND) are shown in parallel. (E) Analysis of WASP expression by intracytoplasmic flow cytometry of WAS1 T cells after transduction with the transiently produced oncoretroviral vectors LWiDN and immunoselection. The percentage of WASP-positive cells within the transduced cells is indicated. Stainings of untransduced WAS T cells (Untr.) and normal donor T cells (ND) are shown in parallel. (F) Analysis of WASP expression by intracytoplasmic flow cytometry of WAS1 T cells after transduction with the lentiviral vectors (PW and WW) and culture for three rounds of feeder stimulation (6-week culture). The percentages of WASP-positive cells within the transduced cells (with PW and WW vectors) are indicated. Stainings of untransduced WAS T cells (Untr.) and normal donor T cells (ND) are shown in parallel. Each of the expression data presented here is representative of three independent experiments.

reached 81% after 18 weeks of culture ($P < 0.01$, t test). In contrast, we observed no selective outgrowth of cells transduced with the GFP-expressing lentiviral vector. These results indicate that transduced WAS patients' T cells have a selective *in vitro* growth advantage over their untransduced counterparts.

Restoration of Proliferation after TCR Stimulation

To investigate whether WASP gene transfer with an optimized oncoretroviral vector or lentiviral vectors resulted in levels of WASP expression sufficient for functional correction, we measured proliferation of transduced T cells after TCR/CD3 stimulation. As shown in Fig. 7A, WAS patients' T cells either untransduced or transduced with a control oncoretroviral vector encoding ΔLNGFR displayed a proliferative defect. For example, in WAS2 T cells, the value corresponding to the half-maximal proliferation was reached with an anti-CD3 mAb dose of 0.5 to 1 μg/ml, whereas in control T cells the same proliferation was reached with the dose of 0.005 μg/ml (Fig. 7A). When we transduced WAS T cells with the WASP-encoding LWiDN oncoretroviral vector and immunoselected, the dose of anti-CD3 mAb required for half-maximal proliferation decreased to approximately 0.02 μg/ml (Fig. 7A), demonstrating that transduction and WASP expression led to a significant improvement in TCR-driven proliferation. The incomplete correction of the proliferative defect could be due to the presence of the residual fraction of untransduced cells that remained in the culture after immunoselection or to heterogeneous levels of expression in transduced cells. In contrast, T cells transduced with either the PW or the WW lentiviral vector and kept in culture for 8 to 14 weeks showed a complete restoration of their proliferative capacity. Indeed the values corresponding to half-maximal proliferation were comparable to those of normal T cells (Fig. 7B). It is important to note that the proliferation of the same T cell lines, immediately after transduction, was intermediate between the proliferation of untransduced cells and the proliferation of normal donor cells (data not

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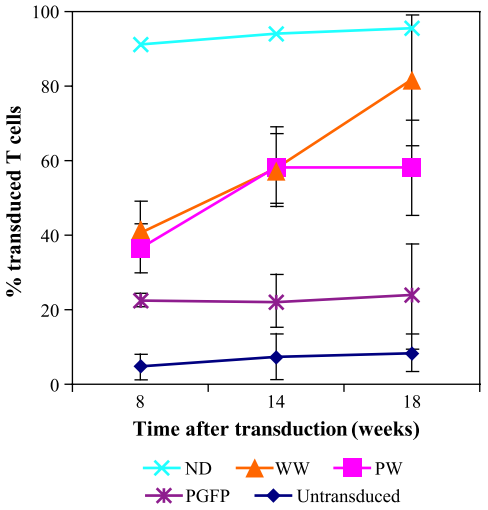

FIG. 6. Analysis of WASP and E-GFP expression in long-term cultures of WAS patients' T cells transduced with lentiviral vectors. Analysis of WASP and E-GFP expression by flow cytometry of WAS T cells after transduction with the lentiviral vectors (PW, WW, and PGFP) at relatively low rates and culture for 7 rounds of feeder stimulation (14-week culture) is shown. The percentages of WASP-positive cells (PW and WW vectors) or GFP-positive cells (PGFP vector) are indicated at 8, 14, and 18 weeks after transduction. WASP stainings of untransduced WAS T cell lines and untransduced normal donor T cell lines (ND) are shown in parallel. Data presented here are the means ± SD of two independent transduction experiments performed on T cell lines from three WAS patients.

shown). Since at this earlier time point the percentage of WASP-positive cells was lower, this finding further indicates that the proportion of WASP-expressing corrected cells increased over time in culture.

Restoration of IL-2 Production and Lipid Raft Levels after TCR Stimulation

Defective production of IL-2 is a possible cause of the T cell unresponsiveness to TCR stimulation observed in WAS patients. To test whether WASP expression following gene transfer could also restore the production of IL-2, we measured the secretion of this cytokine by ELISA in WAS T cell lines before and after WASP gene transfer. Compared to normal controls, untransduced WAS T cells displayed a profound defect in IL-2 production after stimulation with anti-CD3 mAb. After transduction with the LWiDN oncoretroviral vector and immunoselection of the ΔLNGFR-positive cells, we measured a significant increase in IL-2 secretion (Fig. 8A). In T cells transduced with the lentiviral vectors (both PGK promoter- and WASP promoter-containing vectors), the IL-2 secretion was restored to normal levels, when measured at late time points after transduction (Fig. 8B). Our results demonstrate that transduction of WAS patients' T cells with oncoretroviral and lentiviral vectors led to a correction of IL-2 production. The levels of lipid rafts present at the cell membrane, as determined by cell surface expression of GM1, may define the susceptibility of a T cell to activation. We have previously shown that the state of unresponsiveness of WAS patient's T cells was associated with a failure to upregulate plasma membrane lipid raft levels after TCR stimulation [14]. Flow cytometric analysis of GM1-bound CtxB–FITC confirmed that T cells from WAS patients have a strongly reduced capacity to upregulate their levels of surface GM1 expression, compared to normal donors' T cells (Figs. 8C and 8D). After transduction with either the WASP-encoding oncoretroviral vector (Fig. 8C) or the WASP-encoding lentiviral vector (Fig. 8D), patients' T cells were able to upregulate surface GM1 expression significantly. Taken together, these data demonstrate that WASP gene transfer corrects the molecular defects underlying the state of unresponsiveness characterizing WAS T cells.

DISCUSSION

Wiskott–Aldrich syndrome is a primary immunodeficiency for which the development of a gene therapy protocol is highly desired, especially for those patients with no HLA-identical sibling donors for HSC-T. The demonstration of T cell correction by WASP gene transfer is a prerequisite for successful gene therapy since the T cell defects are the main cause for the severe immunodeficiency affecting WAS patients [35]. WASP gene transfer into peripheral T cells could prove to be an effective approach for improving substantially the clinical status of WAS patients. The reinfusion of corrected naïve T cells would be particularly recommended for patients with severe T cell defects who require a rapid reconstitution of their T cell functions. Alternatively, reinfusion of transduced HSC could prove to be the approach of choice for patients with severe defects in both T cells and platelets. Third-generation lentiviral vectors are more efficient than oncoretroviral vectors in transducing hematopoietic cells because their integration in the genome of the host cell is mitosis-independent [36]. Furthermore, they can drive robust and long-term transgene expression that can eventually be regulated by the insertion of specific promoters or regulatory elements [33,34]. In the present study, we tested lentiviral vectors encoding WASP cDNA under the control of either the ubiquitous human PGK promoter or a DNA fragment of the human WASP promoter. Results from gene transfer in patients' PBMC and untransformed T cell lines using these vectors were compared to those obtained with oncoretroviral vectors encoding WASP under the control of viral promoters.

Using a transient transfection protocol to produce both oncoretroviral and lentiviral vectors, we were able to overcome the previously reported limitation of poor infectivity of WASP-encoding oncoretroviral vectors produced from stable packaging cell lines [14]. Indeed, our data suggest that ectopic overexpression of WASP in the stable Am12 packaging cells, which do not normally express WASP, could interfere with correct virus assembly and budding. Interestingly, in the stable producer clones,

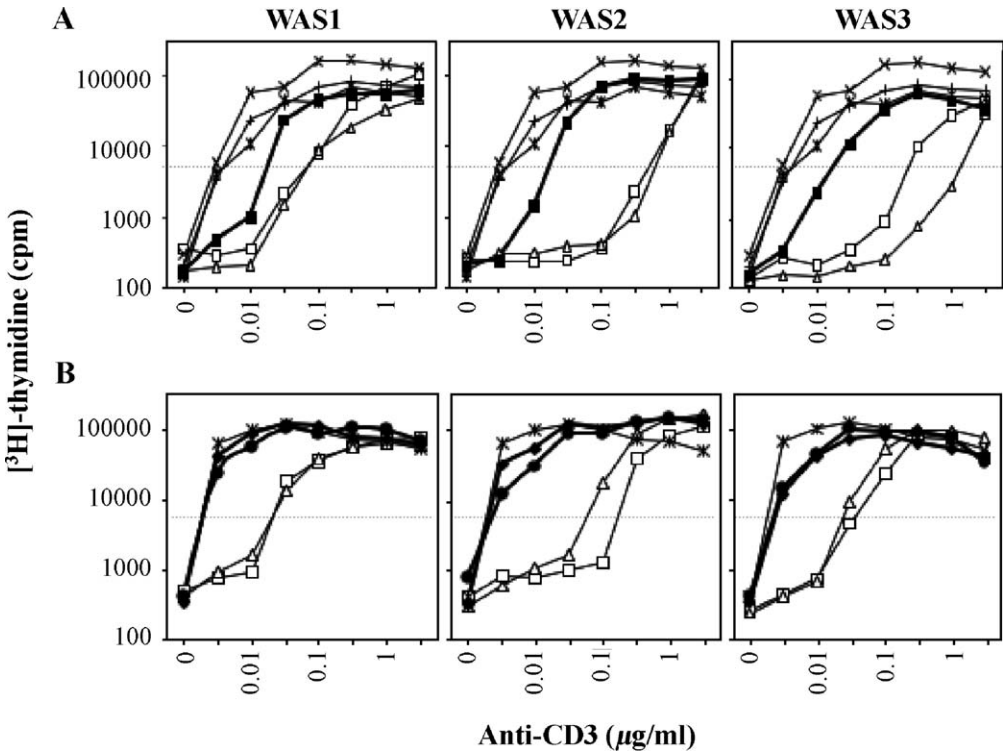

FIG. 7. Cell proliferation after anti-CD3 mAb stimulation. (A) The proliferation of T cells from the three WAS patients transduced with the LWiDN oncoretroviral vector and immunoselected (black squares) was measured after 72 h stimulation with the indicated doses of immobilized anti-CD3 mAb and compared to the proliferation of the T cells from the same patients either untransduced (white squares) or transduced with the pRRV-IRES/ΔLNGFR control vector and immunoselected (white triangles). In parallel, the proliferation of T cells from three normal donors is shown (+, x, *). One representative of five experiments is shown. (B) The proliferation of the untransformed T cell lines from the three WAS patients transduced with the PW (black circles) or WW (black lozenges) lentiviral vectors and cultured for 14 weeks after transduction was measured after 72 h stimulation with the indicated doses of immobilized anti-CD3 mAb. These proliferative responses were compared to those of T cells from the same patients either untransduced (white squares) or transduced with the PGFP control vector (white triangles) cultured under the same conditions. In parallel, the mean proliferation of T cells from two normal donors is shown (*). One representative of three experiments is presented.

the secretion of the gag–pol protein appeared to be normal, whereas the envelope protein was absent from the supernatants, suggesting that envelope-defective particles were produced. A possible explanation for the effect of WASP overexpression in the producer clones is the fact that WASP regulates F-actin polymerization [4] and lipid raft dynamics [14], which are both essential for retrovirus transport, assembly, and budding [37,38]. Indeed, a change in the morphology of the Am12 producer clones with high WASP expression was observed, suggesting a potent effect on the cytoskeleton organization of these cells (data not shown).

Lentiviral vectors proved to be more efficient than oncoretroviral vectors in transducing WASP-deficient patients' T cells, which were prestimulated with anti-CD3 and anti-CD28 mAbs, plus IL-2 and IL-7. In addition, following prestimulation with low doses of IL-2 and IL-7 without TCR activation, lentiviral vectors were found to transduce patient's PBMC at high rates while maintaining their naïve phenotype. These data are consistent with recent reports showing that lentiviral vectors can efficiently transduce naïve T lymphocytes in the absence of cell proliferation [36,39–41]. Preservation of the pool of WASP-deficient naïve T cells would be highly desirable in a clinical setting for long-term restoration of T cell functions. With both vector types, no toxic effects were observed in the transduced T cell lines maintained in culture for at least 3 months. Rather, a substantial increase in the percentage of WASP-positive cells was detected in T cell lines transduced with the WW vector and to a lesser extent with the PW vector. This observation shows that T cells transduced with the lentiviral vector containing the autologous WASP promoter have a selective growth advantage *in vitro*. It is very unlikely that this selective growth advantage would reflect the outgrowth of a few T cell clones as a consequence of insertional mutagenesis. Indeed, the progressive selective advantage was observed in six T cell lines corresponding to two independent transduction experiments. In addition, these T cell lines were dependent on restimulation with irradiated allogeneic cells and IL-2, indicating that they were not transformed. This *in vitro* selective growth advantage may

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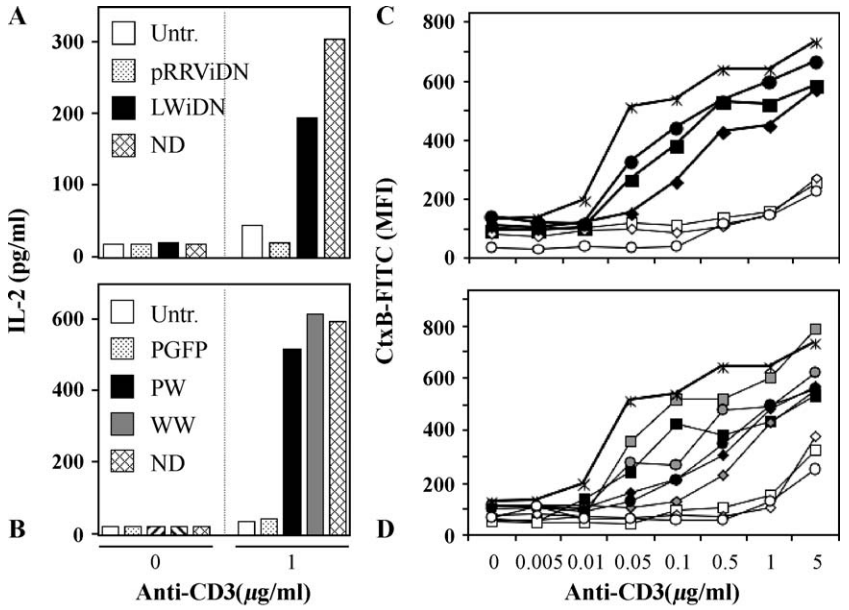

FIG. 8. Cytokine production and surface lipid raft upregulation in transduced WAS patients' T cells after anti-CD3 mAb stimulation. (A) IL-2 production by WAS3 T cells that were either untransduced or transduced with the LWiDN oncoretroviral vector (see key) was measured by ELISA 24 h after anti-CD3 mAb stimulation (1 μg/ml). In parallel, IL-2 production by WAS3 T cells transduced with the control pRRV-IRES/ΔLNGFR vector and control T cells is shown (see key). (B) IL-2 production by WAS1 T cells that were either untransduced or transduced with the PGFP control vector or the WASP-encoding PW and WW lentiviral vectors and cultured for 14 weeks after transduction (see key). IL-2 secretion of normal donor T cells is shown as control (see key). One representative of three experiments is presented. (C and D) The surface levels of the lipid raft marker GM1 were measured by flow cytometric analysis of the MFI of GM1-bound CtxB–FITC on T cell lines that were stimulated for 72 h with the indicated concentrations of immobilized anti-CD3 mAb. (C) Surface GM1 levels in T cells from controls (*) and WAS patients before (empty symbols) and after (filled symbols) transduction with the LWiDN oncoretroviral vector and immunoselection. (D) Surface GM1 levels in T cells from controls (*) and WAS patients before (empty symbols) and after transduction with the PW and WW lentiviral vectors (black and gray symbols, respectively). One representative of two experiments is presented.

reflect the potential *in vivo* growth advantage for WASP-corrected cells, which would be crucial for the success of a gene therapy protocol, as highlighted previously in SCID-X1 and ADA-SCID [19–21].

Under our experimental conditions, transduction with the transiently produced oncoretroviral and lentiviral vectors containing constitutive promoters resulted in WASP expression levels comparable to those of normal control T cells. In addition, the use of a lentiviral vector containing the WASP autologous promoter lead to physiological levels of WASP expression in WAS patients' T cells. Of importance for safety issues is the observation that normal WASP expression levels were obtained after transduction with a relatively low concentration of lentiviral vectors, resulting in the integration of low numbers of vector copies per cell (1 to 3). Transduction of a set of cell lines with lentiviral vectors encoding E-GFP under the control of the ubiquitous PGK promoter or the autologous WASP promoter indicates that the WASP promoter fragment leads preferentially to transgene expression in hematopoietic cells. It remains to be defined if the WASP promoter can lead to regulated expression of vector-derived WASP, which could prove to be important for differentiation and function of hematopoietic cells. In conclusion, the WW vector could be the vector of choice for a gene therapy approach in WAS patients, not only for its efficacy, but also for safety issues.

Previous results indicated that after oncoretroviral vector-mediated gene transfer of WAS T cells, TCR-driven proliferation and IL-2 production were not completely restored, possibly because of limited WASP expression [14,27,28]. Here, we analyzed untransformed WAS T cells in which the expression of WASP was restored to normal levels after transduction. When intermediate to high doses of anti-CD3 mAb were used, proliferation was restored to normal levels after oncoretroviral vector-mediated gene transfer. However, at the lowest doses of stimulation tested, correction of the proliferative defect was not achieved in the cells transduced with the oncoretroviral vector. This might be due to a fraction of untransduced cells present in the T cell lines. Alternatively, the LTR promoter, which is activation-dependent, might not be able to drive finely tuned regulation of WASP that could be required for complete defect correction. In contrast, after WASP gene transfer with both lentiviral vectors, the proliferative defect was fully corrected in T cells activated with different doses of anti-CD3 mAb. Consistently, production of IL-2 after TCR-mediated stimulation was also

fully corrected in the T cells transduced with the lentiviral vectors. We previously showed that the molecular defects of WAS T cells include defects in lipid raft levels and reorganization during immunological synapse assembly, which could account for their unresponsiveness [14]. In addition to correcting the proliferative defects of WAS T cells, transduction with either type of vector resulted in a close to normal upregulation of lipid raft levels at the surface of WAS T cells, following TCR stimulation. These data demonstrate that WASP gene transfer leads to the correction of the molecular defects responsible for the functional impairment of WAS patients' T cells.

Overall, this study indicates the advantages of lentiviral vectors over oncoretroviral vectors for gene correction in WASP-deficient T cells. Third-generation lentiviral vectors transduced WAS T cells at higher rates compared to oncoretroviral vectors and no immunoselection was required, rendering unnecessary the insertion of a marker transgene. We also demonstrated that the use of a cytokine-based prestimulation supports both efficient transduction and maintenance of a naïve phenotype in fresh patients' T cells. Within the lentiviral vector backbone, the WASP autologous promoter appeared to drive preferential expression in hematopoietic cells and normal levels of WASP expression in WAS patients' T cells, leading to complete correction of their *in vitro* function and *in vitro* growth advantage. Importantly, this correction was achieved with a relatively low dose of vector and low number of integrated copies. The efficacy and safety of the lentiviral vectors developed in the present study will be further tested and validated in the WASP-KO mouse preclinical model using either T cells or HSC from WASP-KO mice as targets for WASP gene transfer.

Materials and Methods

Patients and cells. The three WAS patients studied here have been described previously [14]. Blood samples from WAS patients were obtained following standard ethical procedures. Untransformed T cell lines were derived from PBMC isolated on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient and generated as described previously [14]. Briefly, PBMC were stimulated at the concentration of 2×10^5 cells/ml in a final volume of 1 ml in 24-well plates by a feeder cell mixture that comprises irradiated allogeneic PBMC (1×10^6/ml), irradiated EBV-transformed JY cells (1×10^5/ml), PHA (1 μg/ml), IL-2 (100 IU/ml), and IL-7 (10 ng/ml). These allospecific T cells were cultured in IMDM with Yssel supplement (Dyaclone, Besançon, France), 10% FCS, and penicillin/streptomycin and expanded with feeder mixture every 2 weeks.

Construction of oncoretroviral vector and packaging cell clones. The LWSDN and LDNSW vectors were derived respectively from the LXSDN vector and its derivative LDNSN vector [42] by ligation of the human WASP cDNA obtained by RT-PCR (kindly provided by Dr. I. Molina) either downstream of the LTR promoter or downstream of the SV40 promoter, respectively, after excision of the *neo*^{R} gene. Am12 amphotropic producer cell lines [43] were generated by *trans*-infection and selected for ΔLNGFR expression with magnetic beads (Dynabeads M-450; Dynal AS, Oslo, Norway). The bulk producer cell lines were cloned by limiting dilution and 50 ΔLNGFR-expressing clones were assayed for their ability to support transduction of healthy donor PBMC (see Transduction of untransformed WAS patients' T cells and cell lines). In parallel, their titer was measured on NIH3T3 cells and the concentration of the MMLV structural proteins env and gag–pol were measured by ELISA. The SFCMM3 (LTR-*tk*/SV40-ΔLNGFR) vector used as control (clone 16) has been described previously [44]. The LWiDN vector was generated from the LZRS backbone and was produced following transient transfection of 293T cells as described previously [45]. Briefly, the human WASP cDNA was subcloned into the pLZR-IRES/ΔLNGFR vector [46]. The *Bam*HI/*Not*I fragment containing the WASP-IRES/ΔLNGFR cassette from the pLZR-WASP-IRES/ΔLNGFR vector was subcloned into the pRRV-IRES/GFP oncoretroviral vector (kindly provided by Dr. M. A. González), by replacing the corresponding IRES/GFP fragment. pRRV-IRES/ΔLNGFR was used as control vector. High-titer oncoretroviral supernatants were obtained as described previously [47] by cotransfection of 293T cells with the WASP-encoding vector together with the MLV gag–pol expression vector (pNGVL-MLV-*gag-pol*) and the MLV amphotropic envelope expression vector pNGVL-4070A-*env*. Oncoretroviral vector supernatants were collected after 48 h and used immediately for cell transduction.

Construction and production of lentiviral vectors. For the construction of third-generation self-inactivating lentiviral vectors, WASP cDNA was isolated from the LWSDN plasmid by digestion with *Bbs*I and *Bam*HI. It was blunted and inserted into the pBluescript II (KS) plasmid digested with *Eco*RV. The PW construct containing the human PGK promoter was obtained by digestion of the WASP cDNA with *Bam*HI and *Sal*I and insertion into the pRRLsin.cPPT.PGK.E-GFP.Wpre plasmid (PGFP) [47] by replacement of the E-GFP cDNA fragment. The construct containing the WASP autologous promoter (WW) was obtained by removal of the PGK promoter and by insertion of the 5′ promoter region (−1580/+33) of human WASP kindly provided by Dr. Morrone [31]. To test the tissue restriction of the WASP promoter, a vector containing E-GFP under the control of the WASP autologous promoter (WGFP) was constructed by replacing the PKG promoter of PGFP with the −1580/+33 fragment of the WASP genomic region. Production of lentiviral vectors was performed as described previously [47]. Briefly, WASP-encoding transfer plasmids were transfected together with the three packaging plasmids (encoding *gag–pol,* VSV-G *env,* and *rev*) into 293T cells by precipitation with $CaCl_2$. After 14 h, culture medium was renewed to start harvesting virus particles. A single 24-h harvest was performed and virus particles were concentrated by ultracentrifugation. Virus concentration was estimated from the measurement by ELISA of the concentration of p24 and for PGFP and WGFP vectors; titer was measured by infection of HeLa cells with serially diluted virus preparations. Average vector infectivity of HeLa cells was 1.0×10^5 and 0.6×10^4 TU/ng of p24 for PGFP and WGFP, respectively.

Transduction of untransformed WAS patients' T cells and cell lines. T cells used for transduction were untransformed lines obtained after one cycle of feeder stimulation as described above. Forty-eight hours prior to transduction, T cells were stimulated with immobilized anti-CD3 mAb (1 μg/ml), soluble anti-CD28 mAb (1 μg/ml), IL-2 (100 U/ml), and IL-7 (10 ng/ml) and seeded at 1×10^6/ml. Alternatively, to test the ability of lentiviral vectors to transduce naïve T cells from WAS patients in the absence of TCR stimulation, PBMC were stimulated according to the method developed previously [36] with the following modification: PBMC were stimulated for 96 h with IL-2 (60 U/ml) combined with IL-7 (5 ng/ml) prior to transduction. Transduction with lentiviral vectors was performed in the presence of Polybrene (8 μg/ml Polybrene; Sigma) at the indicated concentration (TU/ml) for PGFP or at a concentration of 100 ng p24/1×10^5 T cells in a volume of 0.5 ml for the PW and WW vectors. Cells transduced with the lentiviral vectors were not purified. Transduction with undiluted preparations of oncoretroviral vectors was performed either by "spinocculation" or by infection on retronectin-coated plates as described previously [48]. Transduced cells were immunoselected for ΔLNGFR expression as described above. To test the tissue

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specificity of the WASP promoter in the context of the lentiviral vectors, hematopoietic (Jurkat, JY, U937, HEL) and nonhematopoietic (HeLa, NIH3T3, 293T) cell lines were transduced with PGFP and WGFP lentiviral vectors in parallel at the concentration of 0.1 ng p24/1 $\times$ 10^5 cells/0.5 ml. After transduction, MFI of E-GFP was analyzed by FACS at the indicated time points. For measurement of copy number of vector integrated per genome, genomic DNA was purified from transduced cells several passages after transduction. DNA was amplified with primers specific for the lentiviral vector backbone using TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA) in the presence of a labeled probe. Real-time analysis was performed using a TaqMan PCR detection device. Dilutions of genomic DNA from a HeLa cell clone containing 11 proviruses per genome was used to calculate the copy number of vector integrated per genome of the transduced cells.

WASP expression analysis. For the Western blot analysis of WASP expression, cell lysates were prepared from 1 $\times$ 10^6 PBS-washed cells in 20 μl lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA) supplemented with 100 μg/ml PMSF and a complete set of protease inhibitors (Roche). After 30 min on ice, lysates were centrifuged and supernatant was resuspended in denaturing SDS–PAGE sample buffer. An aliquot of the lysate was used for total protein concentration determination with the BCA protein assay (Pierce, Rockford, IL, USA) to normalize each sample. Following SDS–PAGE resolution, proteins were transferred onto nitrocellulose membranes, stained with Ponceau red to control correct loading and transfer, and then incubated with anti-WASP mAb 5A5 ascites [49] diluted 1:2000 in 5% milk in TBS–Tween 20 (0.05%). HRP-coupled Abs (Dako A/S, Denmark) were used at the dilution of 1:2000 as secondary Abs and detection was performed with the ECL (Amersham Pharmacia Biotech, Little Chalfont, England) detection system. Where indicated, quantification of WASP expression from Western blots was done using a Typhoon 8600 densitometer (Molecular Dynamics, Sunnyvale, CA, USA). WASP expression was also analyzed by FACS after intracytoplasmic staining. Briefly, cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences Pharmingen, San Diego, CA, USA), blocked with 5% normal rabbit serum, and incubated with 5 μg/ml purified anti-WASP mAb 5A5 [49] for 20 min at 4°C. After washing, 5 μg/ml PE-conjugated goat anti-mouse IgG Abs (Southern Biotechnology Associates, Birmingham, AL, USA) were incubated with the cells for 15 min at 4°C. Stained cells were analyzed on a FACScan flow cytometer using the CellQuest software (BD Biosciences).

Proliferation, cytokine production, and cell surface GM1 expression. For the proliferation assay, resting T cells (9 to 14 days post-stimulation with feeder cell mixture) were harvested, centrifuged, and incubated overnight at 2 $\times$ 10^6 cells/ml in fresh medium without cytokine. They were plated at 1 $\times$ 10^5 cells per well in 96-well flat-bottom plates precoated with the indicated dose of anti-CD3 mAb. After 72 h, cells were labeled with [3H]thymidine for 18 h. Thymidine incorporation was measured in triplicate by liquid scintillation counting. Cytokine levels were measured by capture ELISA in the supernatants of cells that had been stimulated with 1 μg/ml immobilized anti-CD3 mAb for 24 h. Capture Abs and biotinylated detection Abs were purchased from BD Biosciences Pharmingen and used according to the manufacturer's instructions. Peroxidase-conjugated streptavidin (Roche Molecular Biomedicals, Mannheim, Germany) was added and developed with tetramethylbenzidine (Fluka Chemie, Buchs, Switzerland) as substrate. For the measurement of surface GM1 expression, resting T cell lines were stimulated with the indicated concentrations of immobilized anti-CD3 mAb (as described for the cell proliferation assays) for 72 h. Surface GM1 expression levels were assessed after staining with FITC-conjugated Cholera toxin (Sigma) and flow cytometry analysis.

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