INTRODUCTION

Wiskott–Aldrich syndrome (WAS) is a rare X-linked primary immunodeficiency caused by the defective expression of the WAS protein (WASP) in hematopoietic cells. Affected patients present both cellular and humoral immunodeficiency, eczema, thrombocytopenia and increased risk of autoimmune disorders and lymphomas. WASP is a cytoplasmic protein that regulates actin polymerization and cytoskeleton reorganization in hematopoietic cells. Absence of WASP causes developmental and functional defects in all immune cells. The formation of the immunological synapse in T cells and T cell receptor (TCR)-dependent activation, cytotoxic activity of CD8+ T cells and natural killer cells, and suppressor activity of naturally occurring regulatory T cells are all impaired in the absence of WASP. The motility, adhesion and migration of B cells are also defective. Additionally, lack of WASP affects podosome formation, motility and T-cell priming by dendritic cells (DCs), as well as podosome and phagocytic cup formation in macrophages. Invariant natural killer T cell functionality, adhesion and migration of neutrophils are also altered in absence of WASP. Moreover, at least in T cells, WASP is also involved in signal transduction. In particular, TCR-dependent nuclear recruitments of nuclear factor of activated T cells (NFAT) in CD4+ T cells, and both NFAT-1 and NFAT-2 in CD8+ T cells are reduced in WAS patients and correlate with defective Th1 cytokine production.

The wide range of cellular defects in WASP-deficient cells results in a complex clinical phenotype in patients. Unless successfully transplanted, life expectancy of severe WAS patients is strongly reduced (approximately 15 years). As bone marrow (BM) transplantation from a mismatched donor is associated with an elevated risk of graft rejection and other related diseases, a gene therapy (GT) approach for WAS treatment has become a suitable alternative. We have previously demonstrated that lentiviral vector (LV)-mediated GT, using a human WAS promoter/cDNA-encoding LV (w1.6W), is safe and effective in inducing WASP expression in many hematopoietic lineages in treated mice. WAS GT (GT mice). Next, we analyzed the reconstitution of DC function of bone marrow-derived DCs (BMDCs). The BMDCs showed efficient in vitro uptake of latex beads and Salmonella typhimurium. When BMDCs from the treated mice (GT BMDCs) and the was−/− mice were injected into wild-type hosts, we found a higher number of cells that had migrated to the draining lymph nodes compared with mice injected with was−/− BMDCs. Finally, we found that ovalbumin (OVA)-pulsed GT BMDCs or vaccination of GT mice with anti-DEC205 OVA fusion protein can efficiently induce antigen-specific T-cell activation in vivo. These findings show that WAS GT significantly improves DC function, thus adding new evidence of the preclinical efficacy of LV-mediated WAS GT.

Keywords: Wiskott–Aldrich syndrome; dendritic cells; gene-corrected cell therapy
functionality by in vitro and in vivo assays. We found that BM-derived DCs (BMDCs) of GT mice were more efficient in the uptake of fluorescently labeled latex beads or Salmonella typhimurium as compared with BMT was−/− BMDCs. Finally, we demonstrated for the first time that by using three different in vivo assays, both GT BMDCs and endogenous DCs can efficiently migrate to draining lymph nodes (LN) and prime antigen-specific T cells. Overall, these data provide evidence of the improvement of DC functionality and contribute to the assessment of the efficacy of WAS GT.

RESULTS

Analysis of WASP-expressing DCs in GT-treated was−/− mice

To generate GT mice, we isolated lineage-negative (lin−) cells from BM of male was−/− mice and transplanted them with a human WAS promoter/cDNA-encoding LV (wit. 6W) at a multiplicity of infection (MOI) of 200. Transduced lin− cells were injected into sublethally irradiated female was−/− recipient mice. In all experiments, we used as controls was−/− mice transplanted with was−/− lin− untransduced BM cells (BMT was−/− mice) and was−/− mice transplanted with wild-type (wt) lin− untransduced BM cells. Treated mice were analyzed 4 months after transplant. Donor cell engraftment was measured by Y chromosome-specific real-time PCR. High engraftment (ranging from 69 to 100%) was achieved both in BM and spleen, with no significant differences among the three groups of mice (Supplementary Figure 1). The proportion of transduced cells was assessed by the presence of integrated viral vector in colonies generated in vitro from lin− cells isolated from BM of reconstituted GT mice 4 months after transplantation. In the cohort of mice analyzed for this study, the proportion of transduced cells was 56 ± 16% (data not shown). The number of LV integrants per donor cell (vector copy number) was measured by vector-specific real-time PCR in total BM, spleen, and BMDCs of GT mice. Total BM cells contained a mean of 3.3 vector copy number per cell and 4.8 in total spleen cells. The presence of WASP+ cells in secondary lymphoid organs of GT mice was analyzed by flow cytometry. Consistent with our previous studies, WASP+ cells in the spleen of GT mice, we found an average of 38% in T cells (CD3+), 23% in B cells (B220+) and 40% WASP+ cells in total myeloid lineage (CD11b+; Figure 1a). We also detected a significant presence of WASP-expressing CD8+ and CD8− conventional DCs (cDCs) in all lymphoid tissues analyzed in GT mice (Figure 1c), demonstrating that cDCs can differentiate from transduced hematopoietic precursors and efficiently colonize secondary lymphoid organs of treated mice. Analyzing WASP relative fluorescence intensity, we found quantitative differences among different immune cells (Figures 1b and d). Such discrepancies have been also detected in immune cells obtained from wt lin− untransduced BM cells, likely reflecting different regulation of WASP expression among different immune cells (Supplementary Figure 2). In addition, the lower WASP expression in immune cells of GT mice compared with BMT wt cells could be due to the use of the human WASP promoter/cDNA, which could lead to a reduced transgene expression in the mouse system. We found that the frequency of CD8+ and CD8− cDCs in spleen, LNs and thymus of GT mice was comparable to BMT wt and BMT was−/− mice (Table 1). In all groups of mice, cDC populations showed low expression of costimulatory ligands CD80 and CD86, consistent with an immature state (Table 1 and Supplementary Figures 3–5). We did not observe any difference in frequency and CD80 and CD86 expression of plasmacytoid DCs (pDCs) among the three groups of mice (data not shown).

Improvement of phagocytic activity of DCs differentiated from BM of GT mice

Phagocytosis of pathogens by DCs is an essential step in the generation of major histocompatibility complex (MHC)/peptide complexes that activate antigen-specific T cells and initiate adaptive immunity. Several lines of evidence showed that WASP has a key role during phagocytosis in macrophages. Indeed, in the absence of WASP, IgG-mediated phagocytosis and apoptotic body uptake are impaired. In particular, WASP has been shown to be involved in the formation of phagocytic cup, an actin-based membrane structure formed at the early stage of phagocytosis upon stimulation with foreign materials. To evaluate whether reconstitution of WASP expression rescues DC functions, we first tested phagocytic activity in DCs differentiated from BM (BMDCs) of GT mice (see purity of BMDCs in Supplementary Figure 6). The percentage of WASP+ BMDCs of GT mice was 30 ± 5% in all experiments (data not shown). BMDCs of BMT wt, BMT was−/− and GT mice were exposed to fluorescent latex beads (3 μm) for 15 min, fixed and labeled with cholera toxin and phalloidin to visualize the plasma membrane and actin, respectively. Internalized beads were identified by single confocal planes and three-dimensional reconstruction of Z-stacks (Figure 2a). In agreement with previous reports, we found that the percentage of internalized beads was strongly reduced in BMT was−/− DCs (80% reduction) as compared with BMT wt control cells (P < 0.001; Figure 2b). Importantly, in GT BMDCs, the percentage of beads+ cells was significantly higher than in BMT was−/− DCs (P < 0.001) and reached 65% of the uptake in BMT wt DCs (Figure 2b). To extend these data to a relevant form of antigen, we measured internalization of bacteria, as WASP-deficient DCs were shown to be defective in processing and presenting bacteria antigens. BMDCs were incubated with green fluorescent protein (GFP)-expressing Salmonella typhimurium (GFP-ST) at three different DC:GFP-ST ratios, and the percentage of CD11c+ /GFP+ cells was analyzed by flow cytometry. Our data showed that GT BMDCs were able to phagocyte GFP-ST more efficiently than BMT was−/− BMDCs, even at low GFP-ST dosages (Figures 2c and d). Overall, these data indicate that GT BMDCs have a significant amelioration of phagocytic function.

Amelioration of in vivo migration of BMDCs of GT mice

WASP is essential for the assembly of actin-rich adhesion structures called podosomes, necessary for directional movement, transcellular diapedesis and migration. Transduction with w1.6W LV has been demonstrated to be effective in restoring podosome formation in immature DCs of was−/− mice or WAS patients. To test the ability of BMDCs of GT mice to migrate in vivo and to enter peripheral LNs, we subcutaneously injected 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled BMDCs (see fluorescence-activated cell sorting (FACS) analysis of CFSE-labeled BMDCs before injection in Supplementary Figure 7a) of BMT wt, BMT was−/− or GT mice into the footpad of C57BL/6 wt mice and collected popliteal LNs 24 h after injection. The percentage of CD11c+/CFSE− cells in the LNs was analyzed by flow cytometry (Figures 3a and b; see raw data in Supplementary Figure 7b). As expected, migratory capacity of BMT was−/− BMDCs was significantly reduced compared with wt controls. Importantly, migration of GT BMDCs was significantly higher than BMT was−/− BMDCs and not statistically different from BMT wt BMDCs, thus showing an increase in the ability to reach peripheral LNs.

BMDCs of GT mice rescue T-cell priming capacity

The ultimate role of DCs in adaptive immunity is to prime naïve T-cell response. It has been established that WASP expression in DCs is required to properly activate CD4+ and CD8+ T lymphocytes. Defective T-cell priming by was−/− DCs is due to multiple defects in the ability to traffic and transport antigens to secondary lymphoid organs and to establish DC–T contacts in LNs. As DC maturation levels can affect T-cell priming, expression of CD80 and CD86 in pulsed BMDCs was evaluated by

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FACS analysis and showed similar maturation profiles among BMT wt, BMT \textit{was}^{-/-} and GT DCs (data not shown). To further investigate maturation capacity, we also evaluated BMDC response to lipopolysaccharide (100 ng ml^{-1}). Twenty-four hours after stimulation, expression of CD80 and CD86 was detected by FACS analysis. Again, we found that GT BMDCs can efficiently upregulate both CD80 and CD86 in response to lipopolysaccharide, similarly to BMT wt and BMT \textit{was}^{-/-} BMDCs (Figure 4). To evaluate whether \textit{WAS} GT reconstitutes DC capacity to prime T cells, we performed three different \textit{in vivo} T-cell activation assays. In the first, C57BL/6 wt recipients were adoptively transferred with CFSE-labeled CD8^{+} T cells specific for ovalbumin (OVA) class-I antigen (OT-I cells). Twenty-four hours later, mice were immunized with OVA class-I-pulsed BMDCs of BMT wt, BMT \textit{was}^{-/-} and GT mice. LNs draining the immunization site were harvested 3 days later to assess proliferation of OT-I cells by flow cytometry. As expected, CFSE dilution profile in mice injected with BMT wt BMDCs showed that T cells underwent several cycles of division and proliferated extensively, whereas T cells primed by BMT \textit{was}^{-/-} BMDCs divided less and failed to expand (Figure 5a). Notably, in mice injected with GT BMDCs, CD8^{+} T-cell proliferation and expansion returned to normal rate. When expressed as percentage of OT-I cells over total number of CD8^{+} T cells, our data show that proliferation of OT-I cells in mice immunized with BMT \textit{was}^{-/-} BMDCs was significantly reduced compared with those immunized with BMT wt BMDCs. On the contrary, OT-I cells primed by GT BMDCs proliferated similarly to those primed by BMT wt BMDCs (Figure 5b). As OVA peptide is not processed by DCs, to investigate GT DCs capacity to prime T cells upon encounter with a full-length antigen, we performed \textit{in vivo} T-cell activation, immunizing mice with OVA protein-pulsed BMDCs. For this assay, wt recipients were adoptively transferred with CFSE-labeled CD4^{+} T cells specific for OVA class-II antigen (OT-II cells). Also in this assay, GT BMDCs showed an improved T-cell priming ability as demonstrated.

\textbf{Figure 1.} Analysis of GT treated \textit{was}^{-/-} mice 4 months after GT. (a-c) Percentage of WASP \textit{+} cells in the indicated cell types. (b-d) Relative fluorescence intensity (RFI) of WASP expression in the indicated cell types, gated on WASP \textit{+} cells. Dots represent values from each mouse. Horizontal bars represent mean value of three different experiments.
by CFSE dilution analysis (Figures 5c and d) and calculation of percentage of OT-II cells over total number of CD4⁺ T cells (Figure 5e) compared with BMT was⁻/⁻ BMDCs. To better evaluate T-cell activation, we detected intracellular interferon (IFN)-γ production in OT-II cells by FACS analysis (Figure 5f). We found that the percentage of IFN-γ producing OT-II cells was significantly higher in mice immunized with GT BMDCs compared with those immunized with BMT was⁻/⁻ BMDCs. As a third approach of in vivo T-cell activation, we immunized GT mice with a recombinant anti-DEC205 OVA fusion protein delivered as naked

Table 1. Frequency and costimulatory ligand expression of cDC populations in spleen, inguinal LNs, thymus of BMT wt, BMT was⁻/⁻ or GT mice, determined by FACS analysis

|          | CD8⁺ cDCs |   | CD8⁻ cDCs |   |
|----------|-----------|---|-----------|---|
|          | %         | CD80 | CD86     | %         | CD80 | CD86     |
| **Spleen** |           |      |          |           |      |          |
| BMT wt   | 0.15 ± 0.04 | 503 ± 82 | 727 ± 42 | 0.40 ± 0.13 | 559 ± 45 | 559 ± 43 |
| BMT was⁻/⁻ | 0.16 ± 0.02 | 470 ± 30 | 873 ± 41 | 0.39 ± 0.02 | 653 ± 90 | 616 ± 83 |
| GT       | 0.11 ± 0.03 | 463 ± 58 | 794 ± 75 | 0.31 ± 0.11 | 584 ± 75 | 526 ± 54 |
| **LNs**  |           |      |          |           |      |          |
| BMT wt   | 0.09 ± 0.03 | 338 ± 17 | 569 ± 46 | 0.20 ± 0.11 | 2172 ± 181 | 1548 ± 204 |
| BMT was⁻/⁻ | 0.10 ± 0.03 | 301 ± 68 | 546 ± 61 | 0.15 ± 0.08 | 2413 ± 199 | 1391 ± 111 |
| GT       | 0.12 ± 0.02 | 340 ± 43 | 523 ± 54 | 0.11 ± 0.03 | 2864 ± 221 | 1429 ± 175 |
| **Thymus** |           |      |          |           |      |          |
| BMT wt   | 0.24 ± 0.06 | 450 ± 6 | 1012 ± 112 |
| BMT was⁻/⁻ | 0.33 ± 0.09 | 486 ± 18 | 839 ± 87 |
| GT       | 0.28 ± 0.06 | 489 ± 37 | 1027 ± 110 |

Abbreviations: BMT, untransduced BM cells; cDCs, conventional dendritic cells; FACS, fluorescence-activated cell sorting; GT, gene therapy; LNs, lymph nodes; MFI, mean fluorescence intensity; wt, wild type. Frequencies are given as percentage on total live cells. CD80 and CD86 MFI is depicted (mean ± s.d.).
DISCUSSION

In this study, we have demonstrated the ability of LV-mediated WAS GT to significantly improve was<sup>−/−</sup> DC functionality, both in vitro and in vivo, by using a LV suitable for clinical application. In particular, we focused our analysis on the evaluation of phagocytosis, migration and priming capacity of DCs, demonstrating for the first time their correction in a preclinical model of WAS GT.

There is increasing evidence that DCs have a key role as regulators of adaptive immunity. DCs exert this function through several mechanisms. For example, during infections, both lymphoid-resident and migratory DCs present pathogen antigens to T cells and trigger their clonal expansion. It is also well established that DCs actively contribute to the maintenance of self tolerance, participating together with stromal cells in negative selection of autoreactive T cells in thymus. Recent studies have reported that was<sup>−/−</sup> DCs are less efficient in inducing antigen-specific T-cell activation in LNs, suggesting a direct contribution of DC functional defects in the pathogenesis of WAS. Interestingly, those studies have highlighted that DC impairment in activating T cells was not only due to delayed migration to LNs, but also to their inefficiency in establishing proper interaction with T cells. For these reasons, the presence of WASP<sup>+</sup> DC in lymphoid organs and correction of DC functional defects is important to increase the effectiveness of WAS GT.

In our work, we report that WASP<sup>+</sup> DCs significantly populate lymphoid organs of GT mice 4 months after transplant. These data demonstrate that GT-corrected DC precursors can efficiently reach lymphoid organs and differentiate in cDCs. This finding is also in line with our previous work that showed the presence of WASP<sup>+</sup> myeloid cells (CD11b<sup>+</sup>) in the spleen of GT mice. Several DC functions require cytoskeleton reorganization and hence WASP recruitment. It has been shown, for example, that WASP-deficient DCs are less efficient in processing and presenting particulate antigens. Such a defect is likely due to impairment of cytoskeleton. During phagocytosis, in fact, WASP and WASP-interacting protein are recruited at the phagocytic cup. In the absence of WASP, formation of phagocytic cup and phagocytosis are impaired in phagocytes. We demonstrated by confocal microscopy and flow cytometry that WAS GT treatment can correct antigen uptake in DCs. In particular, we showed that GT DCs are more efficient in uptake beads and bacteria in vitro as compared with BMT was<sup>−/−</sup> control. The efficiency of T-cell priming depends on DC capacity to home to lymphoid organs and form contact with T cells. We injected BMDCs from treated mice into C57/BL-6 wt mice and evaluated, by flow cytometry, the percentage of cells that reached draining LNs. Our data show an increase in migratory capacity of GT BMDCs compared with BMT.

DNA<sup>48,49</sup> This vaccine allowed the targeting of full-length OVA protein to DC in vivo through the DEC205 receptor.<sup>50</sup> BMT wt, BMT was<sup>−/−</sup> and GT mice were adoptively transferred with OT-I cells followed by immunization with anti-DEC205 OVA DNA. To evaluate DC ability to cross-present OVA antigen in vivo, we measured the proliferation and IFN-γ production by OT-I cells in inguinal LNs, at day 3. We found similar expansion of OT-I cells among the three groups, indicating that, at this antigen dose, there are no major differences in T-cell activation (data not shown). However, the proportion of OT-I cells that produced intracellular IFN-γ was impaired in BMT was<sup>−/−</sup> mice and significantly improved in GT mice, indicating a rescue of cross-presentation capacity by endogenous DCs upon GT (Figure 6). Together, these experiments indicate that reconstitution of WAS expression in was<sup>−/−</sup> DCs by GT is functionally relevant, as it rescues migration to LNs and T-cell priming.

Figure 3. Amelioration of in vivo migration of BMDCs of GT mice. (a) 0.5–2 × 10<sup>6</sup> CFSE-labeled BMDCs from BMT wt, BMT was<sup>−/−</sup> and GT mice were injected into the footpad of C57BL/6 wt mice. Single cell suspensions of popliteal LNs were stained for CD11c and analyzed by flow cytometry. Numbers represent CFSE<sup>+</sup>/CD11c<sup>+</sup> cells, gated on live cells. (b) Numbers of BMT wt, BMT was<sup>−/−</sup> and GT-migrated DCs were normalized to BMT wt-migrated DCs and expressed as percentage. BMT wt (black); BMT was<sup>−/−</sup> (white); GT mice (gray). Bars represent the means ± s.e.m. of three different experiments with three mice per group (n.s., not significant; *P < 0.05, **P < 0.005 Student’s t-test).

Figure 4. Expression of CD80 and CD86 in BMDCs of GT, BMT wt and BMT was<sup>−/−</sup> mice upon lipopolysaccharide challenge, evaluated by flow cytometry (RFI, relative fluorescence intensity; mean ± s.d.).
was\textsuperscript{−/−} BMDCs. When we injected BMDCs pulsed with OVA peptide, we observed increased epitope-specific T-cell priming, measured as CD8\textsuperscript{+} T-cell proliferation. Similarly, we also detected significant T-cell activation upon immunization with full-length OVA protein-pulsed BMDCs. Finally, we reported that IFN-\textgamma production by T cells in GT mice in which endogenous DCs were specifically targeted with anti-DEC205 OVA was improved compared with BMT was\textsuperscript{−/−} mice.

Altogether, these data demonstrate not only that GT BMDCs migrate better to draining LNs, but also that they can effectively reach T-cell areas and stably interact with T cells.

Previous studies on the efficacy of WAS GT have shown significant amelioration of TCR-dependent proliferation, cytokine release, actin polarization at the immunological synapse of T cells, B-cell migration, podosome formation and \textit{in vitro} motility of DCs and reduction of colon inflammation in treated was\textsuperscript{−/−} mice.\textsuperscript{34-38} A recent study has demonstrated the efficacy of a retroviral vector-mediated GT clinical trial for WAS.\textsuperscript{39} Sustained expression of WAS protein and functional correction in T, B, natural killer cells and monocytes was shown in two treated patients, resulting in improved clinical conditions. However, many concerns have arisen regarding the safety of retroviral vector-mediated GT. This study adds new data on the efficacy of a LV-mediated GT for WAS, showing for the first time improvement in phagocytic capacity, \textit{in vivo} migration and T-cell priming of was\textsuperscript{−/−} DCs after GT. Importantly, the w1.6W LV represents a vector suitable for clinical application, as demonstrated by long-term safety and efficacy studies.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 was\textsuperscript{−/−} mice were kindly provided by KA Siminovitch.\textsuperscript{34} C57BL/6 wt mice and OVA specific, MHC class II-restricted, TCR transgenic OT-II mice were purchased from Charles River Laboratories Inc. (Calco, Italy). These mice were housed under specific pathogen-free conditions and treated according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute (IACUC 318). OVA specific, MHC class I-restricted, TCR transgenic OT-I mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). CD45.1 congenic C57BL/6 (a gift from...
Figure 6. DCs of GT mice rescue T-cell priming capacity upon DNA vaccination. BMT wt, BMT \( \text{was}^{+} \) or GT mice were adoptively transferred with \( 1 \times 10^{6} \) OVA class I specific CD8+ T cells, followed by immunization with recombinant DEC205-OVA protein delivered as naked DNA. Inguinal LNs were collected 3 days later. Percentage of IFN-\( \gamma \) expressing OT-I cells evaluated by flow cytometry. Each symbol represents an individual mouse; horizontal lines indicate mean values (n.s., not significant; * \( P < 0.05 \), Student’s t-test).

Pierre Guermonprez, Institut Curie, Paris, France) were bled to OT-I mice to obtain OT-I/CD45.1. Animal care and treatment of OT-I mice were conducted in conformity with institutional guidelines, in compliance with national and international laws and policies (European Economic Community Directive 86/609; OJL 358; 12 December 1987).

Cells
Four -five months after transplantation, mice were killed and total BM cells were collected. BMDCs were differentiated in vitro using culture medium containing Fms-like tyrosine kinase 3 ligand. DCs were used for experiments between day 7 and 9 when expression of CD11c was higher than 80%. BMDCs used in our experiments were more than 90% CD11c+ and, among those, 30% were pDCs (B220+PDCA-1+). For negative selection using a MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Gene therapy
Lineage marker-depleted BM (lin-) cells were purified from 8- to 12-week-old C57BL/6 wt and C57BL/6 \( \text{was}^{+} \) male mice, using the hematopoietic progenitor enrichment kit (Stem Cell Technologies Inc., Vancouver, Canada). The lin- cells were cultured overnight and transduced as previously described. Transduction was performed by culturing \( 1 \times 10^{6} \) lin- cells in the presence of \( 2 \times 10^{6} \) per ml infectious viral genomes (gi) of the \( \text{w}1.6 \) LV (MOI = 200) for 12 h. The \( \text{w}1.6 \) LV is a self-inactivating LV encoding for the human \( \text{WAS} \) cDNA under the control of a 1.6 kb fragment of the autologous proximal \( \text{WAS} \) promoter. After transduction, lin- cells (0.25 \( \times 10^{5} \) cells per mouse) were transferred intravenously into sublethally irradiated (700 rad) 6- to 8-week-old C57BL/6 \( \text{was}^{+} \) female mice. Donor cell engraftment was evaluated by Y chromosome-specific real-time PCR on genomic DNA extracted from total BM cells. To calculate the percentage of transduced cells in reconstituted GT mice, colonies were generated from \( 5 \times 10^{5} \) lin- BM cells by culturing in methylcellulose medium (MethoCult M3434; Stem Cell Technologies) for 10 days. Then, lentivirus-directed real-time PCR was performed on genomic DNA extracted from each single colony.

Real-time PCR analysis
Genomic DNA was extracted from colony-forming units in culture and total BM cells with a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The number of LV copies integrated per genome was assessed by real-time PCR in separate reactions using LV-specific primers (forward, \( 5'-\text{TACTGACGCTCTCGCACC-3'} \); reverse, \( 5'-\text{TCTTGAGCAGGACCTG-3'} \); probe, FAM-\( 5'-\text{ACTTCTCTCTCTTAGCT-3'} \) and \( \beta\)-actin-specific primers (forward, \( 5'-\text{AGAGGAATCGTGCGTGAC-3'} \); reverse, \( 5'-\text{CAATTAGTAGGACCTGCCCGT-3'} \); probe, VIC-\( 5'-\text{CACTGCCGATCCTCTTTCCTCCC-3'} \). Y chromosome-specific real-time PCR was performed with the following primers: forward, \( 5'-\text{GGGCCCACTGAAGATGAC-3'} \); reverse, \( 5'-\text{TCCAGCTGCATTCCACGCT-3'} \); probe, 6-FAM-\( 5'-\text{TGAGGGCAACTTGTTGCCCAGC-3'} \). Results were normalized for the amount of genomic DNA measured by \( \beta\)-actin amplification. The percentage of Y chromosome-bearing cells was determined by interoperation on a standard curve with known female/male ratios. All reactions were performed according to the manufacturer’s instructions and analyzed with an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA).

Flow cytometry
Four to five months after transplantation, BM, spleen, inguinal LNs and thymus were isolated from treated mice. Single cell suspensions were incubated with anti-CD3 (17A2), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD11b/Mac1 (M1/70), anti-CD11c (HL3), anti-CD45 (30F11), anti-CD45R/B220 (RA3-682), anti-CD45.1 (A20), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-I-A\( \beta \) (AF6-120.1), anti-IFN-\( \gamma \) (XM1.2) and anti-DC1c (eBio 972, eBioscience, San Diego, CA, USA) all purchased from BD Pharmingen (San Diego, CA, USA), unless otherwise indicated. Intracytoplasmic detection of human and murine \( \text{WAS} \) was performed using the anti-WASP antibody 503 (a kind gift of Professors H Ochs and LD Notarangelo) after fixation and permeabilization of the cells using Cytotox/Permeabilizer kit (BD Pharmingen).

Phagocytic assays
To measure phagocytosis by immunofluorescence, DCs were pulsed for 15 min with Alexa 488-coupled latex beads, followed by extensive washing. Cells were seeded on poly-L-lysine-coated glass coverslips and fixed in 2% paraformaldehyde for 10 min at room temperature. Cells were labeled with rhodamine-conjugated phalloidin in phosphate-buffered saline 0.2% bovine, 0.05% saponin and Alexa 633-coupled cholera toxin. Images were acquired using a Zeiss confocal microscope (Arese, Milano, Italy). To create three-dimensional images, 20 confocal planes on the zed axis (0.2 \( \mu m \) were reconstructed using Velocity 5.5 (Perkin Elmer Inc., Waltham, MA, USA). For quantification of uptake, only those cells that contained a bead surrounded by plasma membrane were scored as positive. pDCs in the preparation were excluded by size and shape. To measure the uptake of Salmonella enterica serovar typhimurium, we used a non-invasive SPI-I (inVA-) strain transduced to express GFP provided by Dr Maria Rescigno (European Institute of Oncology, Milan, Italy). BMDCs were incubated for 15 min at 37 °C in the presence of three increasing dilutions of bacteria. The cells were then fixed in paraformaldehyde for 10 min at room temperature. After two washes with phosphate-buffered saline 1 mM glycine, the cells were extensively washed with cold phosphate-buffered saline and immediately analyzed by flow cytometry. The uptake was measured on the population of CD11c+ cells excluding B220+ pDCs. The percentage of phagocytosis was measured by the number of GFP+ cells/total number of cells.

In-vivo migration assay
BMDCs were harvested at day 8 and labeled with 2 \( \mu m \) of CFSE (Molecular Probes, Eugene, OR, USA) according to the manufacturer’s instructions. After labeling, \( 5 \times 10^{6} \) to \( 2 \times 10^{6} \) cells, depending on the experiments, were subcutaneously injected into the footpad of C57BL/6 wt hosts. To quantify the number of migrating DCs, popliteal LNs were collected 24 h after injection and single cell suspensions were obtained by digestion with Collagenase D (1.6 mg ml\(^{-1}\); Roche, Mannheim, Germany) and DNase I.
(0.1 mg ml⁻¹, Roche). The absolute number of CFSE⁻/CD11c⁺ cells was quantified by flow cytometry by acquiring all cells in each sample.

**T-cell activation assays**

To test the capacity of OVA class-I-pulsed BMDCs to prime epitope-specific T cells, CD8⁻ cells were isolated from spleen and LN of OT-1/CD45.1⁻ mice by negative selection using a MACS isolation kit. CFSE-labeled OT-I cells of 1.5 × 10⁶ were injected intravenously into C57BL/6 (CD45.2) wt host. After 24 h, 5 × 10⁵ BMDCs loaded with OVA peptide (150 µg ml⁻¹) were injected into the footpad. Three days after BMDC injection, popliteal LNs were collected and single cell suspension was stained using anti-CD8 Pe-Cy5 and anti-CD45.1 PE (eBioscience). The percentage of CD8⁻/CD45.1⁺ cells over total CD8⁻ cells and the CFSE dilution profile were analyzed by flow cytometry.

To test the capacity of full-length OVA protein-pulsed BMDCs to prime epitope-specific T cells, CD8⁻ cells were isolated from spleen and LN of OT-II/CD45.2⁻ mice by negative selection using a MACS isolation kit. CFSE-labeled OT-II cells of 1.5 × 10⁶ were injected intravenously into C57BL/6 (CD45.1) wt host. After 24 h, 5 × 10⁵ BMDCs loaded with OVA protein (150 µg ml⁻¹) were injected into the footpad. Three days after BMDC injection, popliteal LNs were collected and single cell suspension was stained using anti-CD8 Pacific Blue and anti-CD45.2 PerCP-Cy5.5. Before intracellular staining, LN suspensions were incubated with Brefeldin A (10 µg ml⁻¹, Sigma-Aldrich, St Louis, MO, USA) and MHC class-I-restricted peptide of OVA (OVA₃₂₃₋₃₃₉; 2 µM) for 4 h. Cells were fixed with formaldehyde 2%, permeabilized with saponin 0.5% and stained with anti-IFN-γ APC. The percentage of CD8⁻/CD45.2⁺ cells over total CD8⁻ cells, CFSE dilution profile and intracellular IFN-γ were analyzed by flow cytometry.

To test the capacity of *in vivo* OVA-targeted DCs to prime epitope-specific T cells, CD8⁻ cells were isolated from OT-1/CD45.1⁻ and i.v.-injected into BMT wt, BMT or GT mice. After 24 h, mice were immunized with 0.2 mg of a plasmid encoding the V-regions of anti-DEC-205/CD205 rat mAb (clone NLDC-145) cloned in scFv format downstream of the third constant domain of human IgG1 (γ₁-CH₃),³² and delivered intradermally using a gene delivery device (BioRad, Hercules, CA, USA). After 3 days, inguinal LNs were harvested and restimulated with Brefeldin A and MHC class-I-restricted peptide of OVA (2 µM) for 4 h, followed by intracellular staining to detect IFN-γ.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)