Dendritic cell functional improvement in a preclinical model of lentiviral-mediated gene therapy for Wiskott-Aldrich syndrome

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Abstract

Wiskott-Aldrich syndrome (WAS) is a rare X-linked primary immunodeficiency caused by the defective expression of the WAS protein (WASP) in hematopoietic cells. It has been shown that dendritic cells (DCs) are functionally impaired in WAS patients and $was^{-/-}$ mice. We have previously demonstrated the efficacy and safety of a murine model of WAS gene therapy (GT), using stem cells transduced with a lentiviral vector. The aim of this study was to investigate whether GT can correct DC defects in $was^{-/-}$ mice. As DCs expressing WASP were detected in the secondary lymphoid organs of the treated mice, we tested the *in vitro* and *in vivo* 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 to mice injected with $was^{-/-}$ BMDCs. Finally, we found that OVA-pulsed GT BMDCs or vaccination 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 lentiviral vector-mediated WAS GT.

Keywords

Wiskott-Aldrich syndrome; Gene therapy; Dendritic cells

INTRODUCTION

Wiskott-Aldrich syndrome (WAS) is a rare X-linked primary immunodeficiency caused by defective expression of WAS protein (WASP) in hematopoietic cells. Affected patients present both cellular and humoral immunodeficiency, eczema, thrombocytopenia, and increased risk of autoimmune disorders and lymphomas.[1] WASP is a cytoplasmic protein

that regulates actin polymerization and cytoskeleton reorganization in hematopoietic cells.[2] Absence of WASP causes developmental and functional defects in all immune cells. The formation of the immunological synapse (IS) in T cells and TCR-dependent activation,[3-5] cytotoxic activity of CD8^{+} T cells and natural killer (NK) cells,[6,7] and suppressor activity of naturally occurring regulatory T cells[8-11] are all impaired in the absence of WASP. The motility, adhesion and migration of B cells are also defective.[12,13]. Additionally, lack of WASP affects podosome formation,[14,15] motility,[16,17] and T cell priming by dendritic cells (DCs),[18-20] as well as podosome and phagocytic cup formation in macrophages.[21,22] Invariant NKT cell (iNKT) functionality[23,24] adhesion and migration of neutrophils[25] 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 NFAT-1 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.[4,26]

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).[27,28] Since bone marrow (BM) transplantation from a mismatched donor is associated with an elevated risk of graft rejection and other related diseases,[29-33] a gene therapy approach for WAS treatment has become a suitable alternative. We have previously demonstrated that lentiviral vector (LV)-mediated gene therapy, using a human WAS promoter/cDNA-encoding LV (w1.6W) is safe and effective in inducing WASP expression in many hematopoietic lineages in treated *was*^{-/-} mice and restoring T cell activation and B cell migration.[34,35] Very little is known about the functional rescue of innate immune cells upon LV-mediated gene therapy. Some reports showed significant amelioration of motility and podosome formation in DCs after gene therapy,[35-39] but the improvement in other important DC functions, such us phagocytosis, *in vivo* migration and priming capacity, has not been described using a clinically suitable vector, such as w1.6W.

Since it is well known that innate immune cells are directly involved in pathogen clearance, their defect is likely to be the main cause of the high susceptibility to infections in WAS patients. Nevertheless, many other aspects of the WAS pathogenesis can be caused by innate immune cell defect. It is in fact documented that innate immune cells, and in particular DCs, as well as providing defense against pathogens, modulate adaptive immune response and contribute to tumor surveillance.[40,41] Recent reports have demonstrated that DC-T synapse formation and T cell priming require WASP-dependent DC functions.[18-20] This evidence implicates that the rescue of T cell function after WAS gene therapy may not be sufficient to fully recover adaptive immune response, unless also DC functionality is also restored. Indeed, correction of DC defect is required to support the efficacy of WAS gene therapy. In the present work, we first evaluated the presence of WASP-expressing DCs in lymphoid organs of *was*^{-/-} mice treated with LV-mediated WAS gene therapy (GT mice). Next, we analyzed the reconstitution of DC functionality by *in vitro* and *in vivo* assays. We found that bone marrow-derived DCs (BMDCs) of GT mice were more efficient in the uptake of fluorescently-labeled latex beads or *Salmonella typhimurium* as compared to 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 (LNs) 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 gene therapy.

RESULTS

Analysis of WASP expressing dendritic cells in gene therapy treated *was*^{−/−} mice

To generate GT mice, we isolated lineage-negative (lin^{−}) cells from BM of male *was*^{−/−} mice and transduced them with a human WAS promoter/cDNA-encoding LV (w1.6W) at a multiplicity of infection (MOI) of 200 (ref. 34,35). 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 (BMT wt mice). Treated mice were analyzed four months after transplant. Donor cell engraftment was measured by Y chromosome-specific real-time PCR. High engraftment (ranging from 69-100%) 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 four 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, VCN) was measured by vector specific real-time PCR in total BM, spleen cells of GT mice. Total BM cells contained a mean of 3.3 VCN/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,[34,35] 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 (RFI), we found quantitative differences among different immune cells (Figure 1b and 1d). Such discrepancies have been also detected in immune cells obtained from BMT wt mice, 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 to 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, lymph nodes 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 bone marrow of GT mice

Phagocytosis of pathogens by DCs is an essential step in the generation of MHC/peptide complexes that activate antigen-specific T cells and initiate adaptive immunity. Several lines of evidence showed that WASP plays a key role during phagocytosis in macrophages. Indeed, in the absence of WASP, IgG-mediated phagocytosis and apoptotic body uptake are impaired.[42,43] 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.[22] To evaluate whether reconstitution of WASP expression rescues DC functions, we first tested phagocytic activity in DCs differentiated from bone marrow (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 minutes, 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 3D 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, since WASP-deficient DCs were shown to be defective in processing and presenting bacteria antigens.[44] BMDCs were incubated with GFP-expressing *Salmonella Typhimurium* (GFP-S.T.) at three different DC:GFP-S.T. ratios, and the percentage of CD11c^{+}/GFP^{+} cells was analyzed by flow cytometry. Our data showed that GT BMDCs were able to phagocyte GFP-S.T. more efficiently than BMT $was^{-/-}$ BMDCs, even at low GFP-S.T. dosages (Figure 2c-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,[21,45,46] necessary for directional movement, trans-cellular diapedesis and migration.[47] Transduction with w1.6W LV has been demonstrated to be effective in restoring podosome formation in immature DCs of $was^{-/-}$ mice or WAS patients.[35,37] To test the ability of BMDCs of GT mice to migrate *in vivo* and to enter peripheral LNs, we subcutaneously injected CFSE-labeled BMDCs (see 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 hours after injection. The percentage of CD11c^{+}/CFSE^{+} cells in the LNs was analyzed by flow cytometry (Figures 3a-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.[17-20] Since DC maturation levels can affect T cell priming, expression of CD80 and CD86 in pulsed BMDCs was evaluated by FACS analysis and showed similar maturation profiles among BMT wt, BMT $was^{-/-}$ and GT DCs (data not shown). To further investigate maturation capacity, we also evaluated BMDC response to LPS (100 ng/ml). 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 LPS, similarly to BMT wt and BMT $was^{-/-}$ BMDCs (Figure 4). In order to evaluate whether WAS gene therapy reconstitutes DC capacity to prime T cells, we performed three different *in vivo* T cell activation assays. In the first, C57BL/6 wt recipients were adoptively transferred with CFSE-labeled CD8^{+} T cells specific for OVA class-I antigen (OT-I cells). Twenty-four hours later, mice were immunized with OVA class-I-pulsed BMDCs of BMT wt, BMT $was^{-/-}$ and GT mice. LNs draining the immunization site were harvested three 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 $was^{-/-}$ 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 $was^{-/-}$ BMDCs was significantly reduced compared to 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). Since OVA peptide is not processed by DCs, in order to investigate GT DCs capacity to prime T cells upon encounter with a full-length antigen, we performed *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 by CFSE dilution analysis (Figure 5c-d) and calculation of percentage of OT-II cells over total number of CD4$^+$ T cells (Figure 5e) compared to BMT $was^{-/-}$ BMDCs. To better evaluate T cell activation we detected intracellular 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 to 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 DNA.[48,49] This vaccine allowed the targeting of full-length OVA protein to DC *in vivo* through the DEC205 receptor.[50] BMT wt, BMT $was^{-/-}$ 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 $was^{-/-}$ 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 WASP expression in $was^{-/-}$ DCs by gene therapy is functionally relevant as it rescues migration to LNs and T cell priming.

DISCUSSION

In this study, we have demonstrated the ability of LV-mediated WAS gene therapy to significantly improve $was^{-/-}$ 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 gene therapy.

There is increasing evidence that DCs play a key role as regulators of adaptive immunity.[40] 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.[51] 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.[52] Recent studies have reported that $was^{-/-}$ DCs are less efficient in inducing antigen-specific T cell activation in LNs,[18-20] suggesting a direct contribution of DC functional defects in the pathogenesis of WAS. Interestingly, those studies have highlighted that $was^{-/-}$ 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$^+$ DC in lymphoid organs and correction of DC functional defects is important to increase the effectiveness of WAS gene therapy.

In our work, we report that WASP[+] DCs significantly populate lymphoid organs of GT mice four 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^{+} myeloid cells (CD11b^{+}) in the spleen of GT mice.[35] 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.[44] Such a defect is likely due to impairment in the uptake capacity. During phagocytosis, in fact, WASP and WASP-interacting protein (WIP) are recruited at the phagocytic cup.[22] In the absence of WASP, formation of phagocytic cup and phagocytosis are impaired in phagocytes.[42] We demonstrated by confocal microscopy and flow cytometry that WAS gene therapy treatment can correct antigen uptake in DCs. In particular, we showed that GT BMDCs are more efficient in uptake beads and bacteria *in vitro* as compared to BMT *was*^{−/−} 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 lymph nodes. Our data show an increase in migratory capacity of GT BMDCs compared with BMT *was*^{−/−} BMDCs. When we injected BMDCs pulsed with OVA peptide we observed increased epitope-specific T cell priming, measured as CD8^{+} 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-γ production by T cells in GT mice in which endogenous DCs were specifically targeted with anti-DEC205 OVA was improved compared to BMT *was*^{−/−} mice.

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

Previous studies on the efficacy of WAS gene therapy 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 *in vitro* motility of DCs, and reduction of colon inflammation in treated *was*^{−/−} mice.[34-38,53] A recent study has demonstrated the efficacy of a retroviral vector-mediated gene therapy clinical trial for WAS.[39] Sustained expression of WAS protein and functional correction in T, B, NK 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 gene therapy. This study adds new data on the efficacy of a LV-mediated gene therapy for WAS, showing for the first time improvement in phagocytic capacity, *in vivo* migration and T cell priming of *was*^{−/−} DCs after gene therapy. 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*^{−/−} mice were kindly provided by K. A. Siminovitch.[54] 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 (SPF) 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. CD45.1 congenic C57BL/6 (a gift from Pierre Guermonprez, Institut Curie, Paris, France) were bred 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 (EEC) Council Directive 86/609; OJL 358; December 12, 1987).

Cells

Four-five months after transplantation, mice were sacrificed and total BM cells were collected. BMDCs were differentiated *in vitro* using culture medium containing Fms-like tyrosine kinase 3 ligand (Flt3L). 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^{+}) (Supplementary Figure 6). BMDC maturation was induced by incubation with LPS (100ng/ml; Enzo Life Sciences International, Exeter, UK) for 24 hours. CD4^{+} or CD8^{+} cells were isolated from spleen and LN suspensions by 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 *was*^{−/−} male mice using the hematopoietic progenitor enrichment kit (Stem Cell Technologies Inc., Vancouver, Canada). Lin^{−} cells were cultured overnight and transduced as previously described.[34] Transduction was performed by culturing 1×10^6 lin^{−} cells in the presence of 2×10^8/ml infectious viral genomes (ig) of the w1.6W lentiviral vector (LV) (MOI=200) for 12 hours. The w1.6W is a self-inactivating LV encoding for the human *WAS* cDNA under the control of a 1.6kb fragment of the autologous proximal *WAS* promoter.[34,37,55,56] After transduction, lin^{−} cells (0.25×10^6 cells/mouse) were transferred intravenously into sublethally irradiated (700 rad) 6- to 8-week old C57BL/6 *was*^{−/−} female mice. Donor cell engraftment was evaluated by Y chromosome-specific real-time polymerase chain reaction (PCR) on genomic DNA extracted from total BM cells. To calculate the percentage of transduced cells in reconstituted GT mice, colonies were generated from 5×10^4 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 (CFU-C) and total BM cells with a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The number of lentiviral vector copies integrated per genome was assessed by real-time PCR in separate reactions using lentiviral vector-specific primers (forward, 5′-TACTGACGCTCTCGCACC-3′; reverse, 5′-TCTCGACGCAGGACTCG-3′; probe, FAM-5′-ATCTCTCTCCTTCTAGCCTC-3′) and b-actin-specific primers (forward, 5′-AGAGGGAAATCGTGCGTGAC-3′; reverse, 5′-CAATAGTGATGACCTGGCCGT-3′; probe, VIC-5′-CACTGCCGCATCCTCTTCCTCCC-3′). Y chromosome-specific real-time PCR was performed with the following primers: forward, 5′-GCGCCCCATGAATGCAT-3′; reverse, 5′-TCCACCTGCATCCCAGCT-3′; probe, 6-FAM-5′-TGAGAGGCACAAGTTGGCCCAGC-3′. Results were normalized for the amount of genomic DNA measured by b-actin amplification. The percentage of Y chromosome-bearing cells was determined by interpolation 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).

Flow cytometry

Four-five months after transplantation, bone marrow, spleen, inguinal lymph nodes and thymus were isolated from treated mice. Single cell suspensions were incubated with anti-CD3 (17A2), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD11b/Mac1 (M1/70), anti-CD11c (HL3), anti-CD45 (30F11), anti-CD45R/B220 (RA3-6B2), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-I-A[b] (AF6-120.1), anti-IFN-γ (XMG1.2), anti-PDCA-1 (eBio 972, eBioscience, San Diego, CA) all purchased from BD Pharmingen (San Diego, CA) unless otherwise indicated. Intracytoplasmic detection of human and murine WASP was performed using the anti-WASP antibody 503 (a kind gift of Profs. H. Ochs and L.D. Notarangelo) after fixation and permeabilization of the cells using Cytofix/Cytoperm kit (BD Pharmingen).

Phagocytic assays

To measure phagocytosis by immunofluorescence, DCs were pulsed for 15 minutes 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 (PFA) for 10 minutes at RT. Cells were labeled with rhodamine-conjugated phalloidin in PBS 0.2% BSA, 0.05% saponin and Alexa 633-coupled cholera toxin. Images were acquired using a Zeiss confocal microscope. To create 3D images, 20 confocal planes on the zed axis (0.2 μM) were reconstructed using Volocity 5.5 (Perkin Elmer Inc, Waltham, MA, US). 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 noninvasive SPI-I *(InvA⁻)* strain transduced to express GFP provided by Dr. Maria Rescigno (European Institute of Oncology, Milan). BMDCs were incubated for 15 minutes at 37°C in the presence of three increasing dilutions of bacteria. The cells were then fixed in PFA for 10 minutes at RT. After two washes with PBS 1 mM glycine, the cells were extensively washed with cold PBS and immediately analyzed by flow cytometry. The uptake was measured on the population of CD11c^{hi} 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 mM of 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) according to the manufacturer's instructions. After labeling, 5×10^5 to 2×10^6 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 hours after injection and single-cell suspensions were obtained by digestion with Collagenase D (1.6 mg/ml, Roche, Mannheim, Germany) and DNase I (0.1 mg/ml, 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-I/CD45.1$^+$ mice by negative selection using a MACS isolation kit. 1.5×10^6 CFSE-labeled OT-I cells were injected intravenously into C57BL/6 (CD45.2) wt host. After 24 hours, 5×10^5 BMDCs loaded with 0.05 nM of MHC class-I restricted peptide of OVA (OVA$_{257-264}$, SIINFEKL) 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, $CD4^+$ cells were isolated from spleen and LN of OT-II/$CD45.2^+$ mice by negative selection using a MACS isolation kit. 1.5×10^6 CFSE-labeled OT-II cells were injected intravenously into C57BL/6 (CD45.1) wt host. After 24 hours, 5×10^5 BMDCs loaded with OVA protein (150 μg/ml) were injected into the footpad. Three days after BMDC injection, popliteal LNs were collected and single cell suspension was stained using anti-CD4 Pacific Blue and anti-CD45.2 PerCP-Cy5.5. Before intracellular staining LN suspensions were incubated with Brefeldin A (10 μg/ml, Sigma-Aldrich, St. Louis, MO) and MHC class-II restricted peptide of OVA ($OVA_{323-339}$) (2 μM) for four hours. Cells were fixed with formaldehyde 2%, permeabilized with Saponin 0.5% and stained with anti-IFN-γ APC. The percentage of $CD4^+/CD45.2^+$ cells over total $CD4^+$ 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-I/$CD45.1^+$ and i.v. injected into BMT wt, BMT *was*$^{-/-}$ or GT mice. After 24 hours, 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),[57] delivered intradermally using a gene delivery device (BioRad, Hercules, CA, USA). After three days, inguinal LNs were harvested and restimulated with Brefeldin A and MHC class-I restricted peptide of OVA (2 μM) for four hours, followed by intracellular staining to detect IFN-γ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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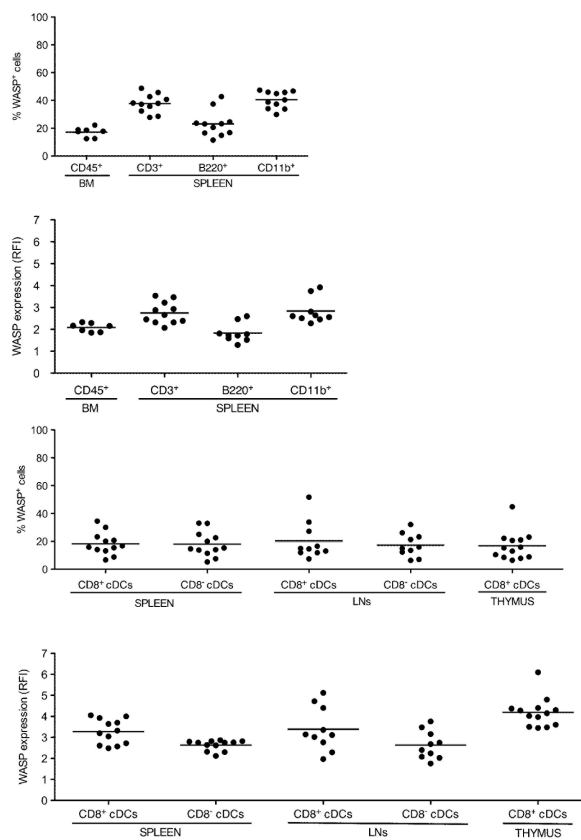
Figure 1.
Analysis of gene therapy treated *was*^{−/−} mice four months after gene therapy. (**a-c**) Percentage of WASP⁺ cells in the indicated cell types. (**b-d**) Relative fluorescence intensity (RFI) of WASP expression in the indicated cell types, gated on WASP⁺ cells. Dots represent values from each mouse. Horizontal bars represent mean value of three different experiments.
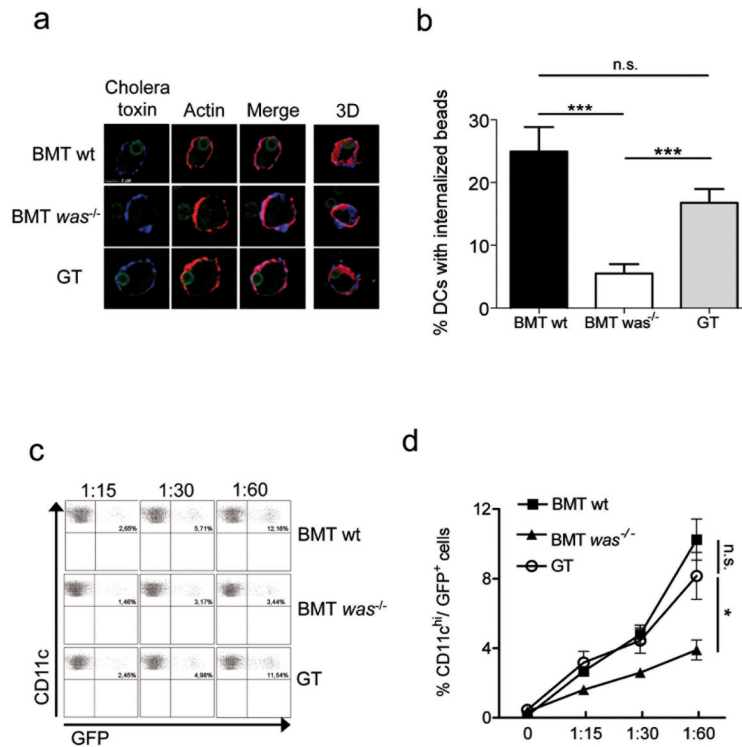
Figure 2.
Improvement of phagocytic activity of DCs differentiated from BM of GT mice. (**a-b**) BMDCs of BMT wt, BMT $was^{-/-}$ and GT mice were incubated with Alexa 488-coupled latex beads for 15 minutes at 37°C, then fixed and labeled with rhodamine-phalloidin and Alexa 633-coupled cholera toxin to visualize F-actin (red) and plasma membrane (blue), respectively. (**a**) Single confocal planes and 3D reconstruction showing internalized beads (BMT wt and GT BMDCs) and external beads (BMT $was^{-/-}$ BMDCs). (**b**) Quantification of phagocytosis. Bars show the percentage of cells that have taken up one or more beads. Mean + SEM of at least 160 cells/group. (**c-d**) BMT wt, BMT $was^{-/-}$ and GT BMDCs were infected with increasing doses of GFP-S.T. for 20 minutes at 37°C. (**c**) Representative dot plots show CD11c^{hi} gated cells. Percentage of CD11c^{hi}/GFP$^+$ cells is depicted. (**d**) Percentages of CD11c^{hi}/GFP$^+$ cells shown as mean of three independent experiments ± SEM (n.s., not significant; SEM, standard error mean; * p<0.05 student t test; ** p<0.005 student t test; *** p<0.001 student t test).
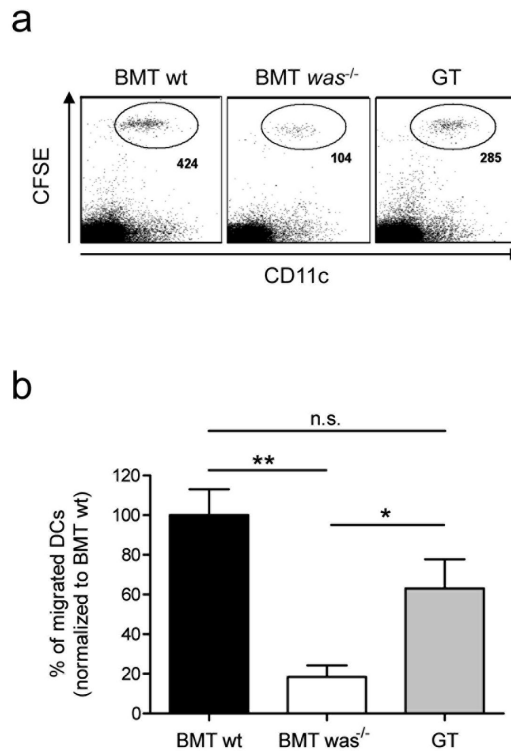
Figure 3.

Amelioration of *in vivo* migration of BMDCs of GT mice. (**a**) 0.5-2×10^6 CFSE-labeled BMDCs from BMT wt, BMT *was*$^{-/-}$ 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$^+$/CD11c$^+$ cells, gated on live cells. (**b**) Numbers of BMT wt, BMT *was*$^{-/-}$ and GT migrated DCs were normalized to BMT wt migrated DCs and expressed as percentage. BMT wt (black); BMT *was*$^{-/-}$ (white); GT mice (gray). Bars represent the means + SEM of three different experiments with three mice per group (n.s., not significant; *p<0.05, student *t* test; **p<0.005 student *t* test; SEM, standard error mean).
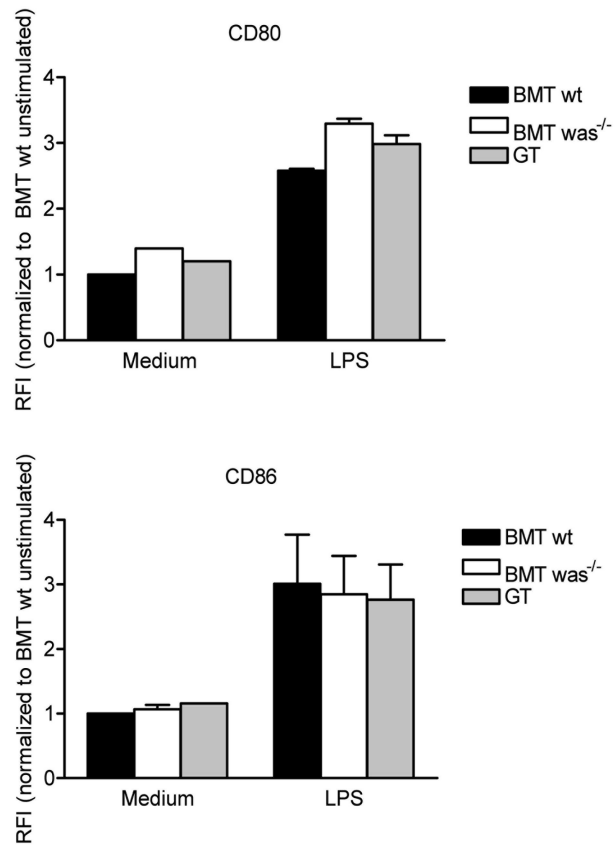
Figure 4.
Expression of CD80 and CD86 in BMDCs of GT, BMT wt and BMT was$^{-/-}$ mice upon LPS challenge, evaluated by flow cytometry (RFI, relative fluorescence intensity; mean ± SD).
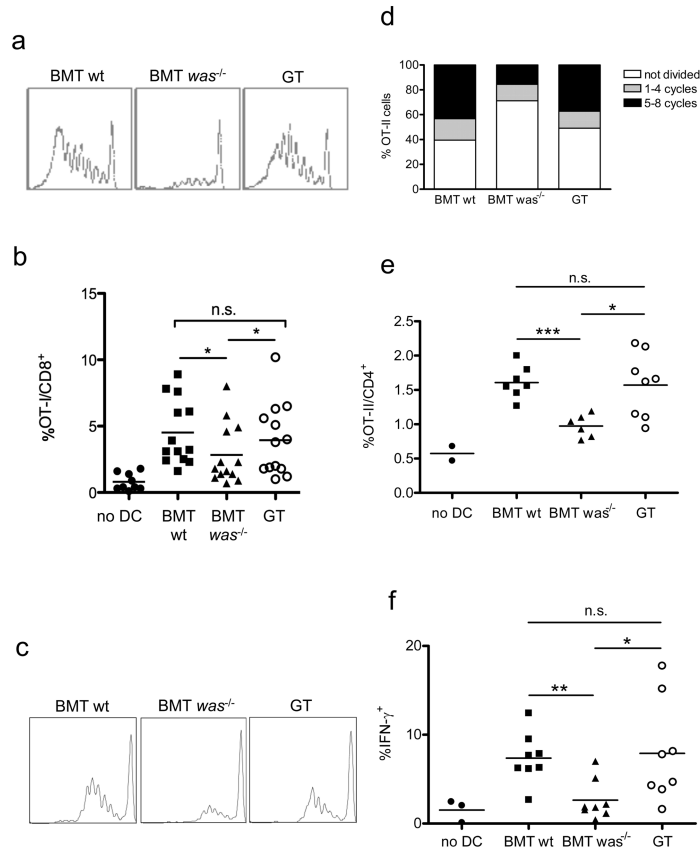
Figure 5.
BMDCs of GT mice rescue T cell priming capacity. (**a-b**) Five $\times 10^5$ BMDCs of BMT wt, BMT $was^{-/-}$ or GT mice were loaded with 0.05 nM of SIINFEKL OVA peptide and injected subcutaneously in C57BL/6 (CD45.2) wt recipient mice that had been transferred with 1.5×10^6 CFSE-labeled OT-I/CD45.1 cells 24 hours before. Draining LNs were collected 3 days after and T cell proliferation was analyzed by flow cytometry. (**a**) Representative histograms showing CFSE dilution profile of transferred OT-I cells, gated on CD8+/CD45.1+ live cells. (**b**) Quantification of T cell expansion expressed as percentage of OT-I cells over the total CD8+ T cell population. Each symbol represents an individual mouse; horizontal lines indicate mean values. (**c-e**) Five $\times 10^5$ BMDCs of BMT wt, BMT $was^{-/-}$ or GT mice were loaded with OVA (150 μg/ml) and injected subcutaneously in C57BL/6 (CD45.1) wt recipient mice that had been transferred with 1.5×10^6 CFSE-labeled OT-II/CD45.2 cells 24 hours before. Draining LNs were collected 3 days later and T cell proliferation and intracellular IFN-γ production was analyzed by flow cytometry. (**c**) Representative histograms showing CFSE dilution profile of transferred OT-II cells, gated on CD4+/CD45.2+ live cells. (**d**) Data obtained from the analysis of CFSE dilution were expressed as the percentage of OT-II cells that remain undivided, that divided one to four times, or that divided five to eight times. (**e**) Quantification of T cell expansion expressed as percentage of OT-II cells over the total CD4+ T cell population. (**f**) Percentage of IFN-γ expressing OT-II cells, evaluated by flow cytometry. Each symbol represents an individual mouse; horizontal lines indicate mean values (n.s., not significant; *p<0.05, **p<0.005, ***p<0.001 student t test).
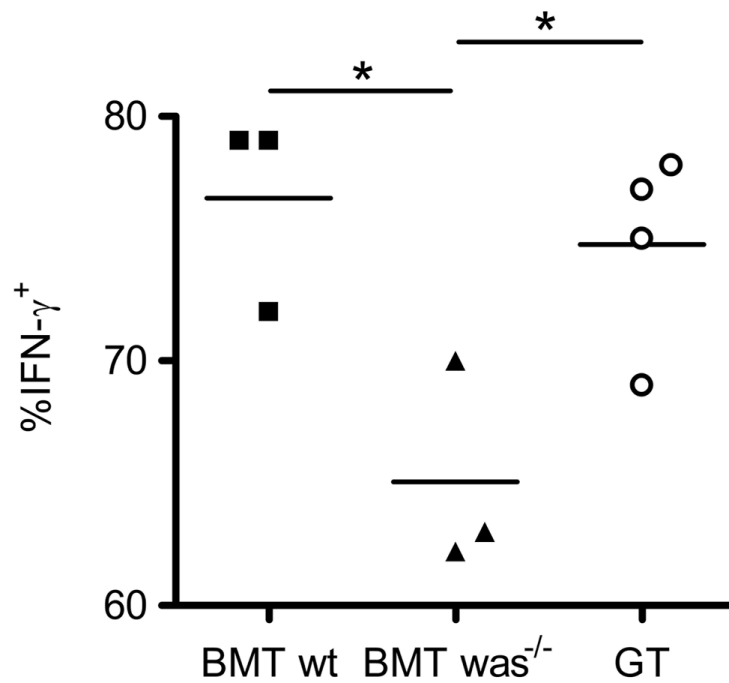
Figure 6.
DCs of GT mice rescue T cell priming capacity upon DNA vaccination. BMT wt, BMT $was^{-/-}$ or GT mice were adoptively transferred with 1×10^6 OVA class-I specific CD8$^+$ T cells followed by immunization with recombinant DEC205-OVA protein delivered as naked DNA. Inguinal LNs were collected three days later. Percentage of IFN-γ 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 t test).

Table 1

Frequency and costimulatory ligand expression of cDCs populations in spleen, inguinal lymph nodes, thymus of BMT wt, BMT *was−/−* or GT mice, determined by FACS analysis. Frequencies are given as percentage on total live cells. CD80 and CD86 MFI is depicted (cDCs, conventional DCs; MFI, mean fluorescence intensity; mean ± SD).

Spleen	CD8+ cDCs			CD8− cDCs		
	%	CD80	CD86	%	CD80	CD86
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
Lymph nodes	%	CD80	CD86	%	CD80	CD86
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	%	CD80	CD86			
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			