Article

Gene Transfer to Dendritic Cells Induced a Protective Immunity against Melanoma

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Lentiviral vectors have shown promises for efficient gene transfer to dividing as well as nondividing cells. In this study, we explored lentiviral vector-mediated, the entire *mTRP-2* gene transfer and expression in dendritic cells (DCs). Adoptive transfer of DCs-expressing mTRP-2 (DC-HR'CmT2) into C57BL/6 mouse was also assessed. Dendritic cells were harvested from bone marrow and functional DCs were proved by allogeneic mixed lymphocyte reaction. Lentiviral vectors were produced by transient transfection of 293T cells. Transduction of DCs was proved by marker gene expression and PCR and RT-PCR amplification. Implantation of the transduced DCs, depletion of immune cells as well as the survival of the mice after tumour challenge were investigated. High efficiency of gene transfer into mature DCs was achieved. The high level expression of the functional antigen (TRP-2) and induction of protective immunity by adoptive transfer of *TRP-2* gene modified DCs were demonstrated. *In vivo* study showed a complete protection of mice from further melanoma cell challenge. In comparison, only 83% of mice survived when mTRP-2 peptide-pulsed DCs were administered, suggesting the generation of specific protection. Together, these results demonstrated the usefulness of this gene transfer to DC approach for immunotherapy of cancer and indicated that using tumour associated antigens (TAAs) for gene transfer may be potentially beneficial for the therapy of melanoma. *Cellular & Molecular Immunology*. 2005; 2(4):281-288.

Key Words: lentiviral vector, gene transfer, dendritic cell, tumour antigen, melanoma

Introduction

Tyrosinase-related protein-2 (TRP-2) is a melanocyte-specific enzyme that catalyses the non-decarboxylative tautomerization of L-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) in the melanin biosynthetic pathway. Similar to several other proteins, such as BAGE, GAGE, MAGE, MART-1, gp100, tyrosinase and tyrosinase related protein-1 (TRP-1), TRP-2 has been identified as a human melanoma-associated antigen (MAAs) (1). The MAA epitope of TRP-2 is a non-mutated peptide and shared by melanoma cells. Active immunisation against this MAA represents an attractive strategy for the therapy of melanoma. However, similar to other MAAs, little is known clinically to use this MAA for optimal therapies to benefit patients (1). Recently, the identification of TRP-2 as a tumour rejection antigen for murine B16 melanoma provides the opportunity for the development of an accurate preclinical model to evaluate new therapeutical strategies (2). Studies using the mouse model so far have produced encouraging results (3, 4).

In the murine models, significant numbers of CTL precursors against murine TRP-2 (mTRP-2) exist in both tumour immunised and naïve mice (1, 2). This indicates that thymic, i.e., central tolerance, to mTRP-2 is not complete and may be a quantitative condition dependent on level of antigen expression and affinity of TCR binding. Hence stimulating the immune system with the antigen from an altered source may break this peripheral tolerance.

It is possible to transfer specific antigens to dendritic cells (DCs), a class of potent professional antigen-presenting cells (APCs) of the immune system to break the immune tolerance and to induce an immune response. DCs are mobile and have very active surface motion, notably macro-pinocytosis, pseudopod extensions and fluttering intervening veils. Maturation of DCs is accompanied by changes in surface membrane activity to the extension/retraction movements effecting cell motility to accelerate their trek to lymphatic vessels and transport to draining lymph nodes. They are able to migrate through tissues to the T lymphocyte

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dependent areas of the draining lymph node and there stimulate antigen specific T cells to proliferate to generate activated progeny. DCs express high levels of MHC class I and class II as well as important adhesion and costimulatory molecules. A significant role that DCs play in the T lymphocyte-DC cluster is believed to be the minimal immune regulatory entity needed to produce a specific cytolytic response (5-7). The interactions between CD40 ligand and CD40 on the $CD4^+$ T cell and the APC, in particular, appear to be significant in the activation of APCs to present antigens and prime $CD8^+$ precursor cells (8). The advent of *in vitro* culture systems to generate large numbers of DCs provides the possibility of *ex vivo* modification of DCs to produce a desired immune response upon adoptive transfer or infusion.

DCs pulsed with MHC class I-binding peptide epitopes have been shown to induce CTL responses against model antigens in mice and provide protection against challenge from tumour cells encoding these antigens (9-11). However, peptide pulsed DCs are reversible, and have been shown to be ineffective by 24 h after pulsing (12). Moreover, repeated stimulations of T cells with peptide-loaded DCs can lead to the emergence of non-lytic $CD4^+$ cells which block the activation of fresh T lymphocytes, leading to immune unresponsiveness (13). Transfection of DCs with plasmid DNA has also been attempted, but was relatively inefficient (14).

Viral vector-mediated gene transfer has the potential to transfer antigen gene to DCs efficiently. At present, retroviral vectors based on murine Moloney leukaemia virus (MMoLV) are the best-characterised viral vehicles used for cell gene transfer. Despite some restrictive factors, several studies have shown successful transduction of DCs, or their precursors, with these vectors (15-17). The most limiting conditions are the low transduction efficiency and the requirement of expensive transduction-enhancement reagents such as RectroNectin (18-20). Other viral vectors used for DC transduction include recombinant avipoxvirus (21), adenovirus (22-23) and HIV-1 based vectors (24). Recombinant adenovirus mediated gene delivery so far appears as the most efficient and popular method (25). Recently, *mTRP-2* was successfully transferred into DCs *via* an adenoviral vector (3). Adenoviruses do not integrate into the host chromosome so the vectors derived from these viruses have negligible oncogenic potential. The dis-advantages are the development of inflammation and the transient expression of the recombinant genes. These effects are believed to be the result of an induced host immune response, specifically the $CD8^+$ cytotoxic T lymphocytes acting against the cells containing the adenovirus antigen (26). Since the HIV-1 vectors do not express any viral proteins, their use should cause no vulnerability to the host cell.

In this study, an HIV-1 based vector was assessed for the delivery of the entire *mTRP-2* cDNA to DCs. The potential effects of the lentivirally-transduced DCs for the induction of anti-melanoma responses in a murine B16 melanoma model as well as the importance of $CD4^+$ and $CD8^+$ T cells were also determined.

Materials and Methods

Tumour cell line

B16-F10, a murine melanoma cell line, was used in this study. For *in vivo* passage, B16-F10 cells (5×10^4 cells in PBS) were injected subcutaneously in the interscapular area of pathogen-free, 5-7 week old female C57BL/6 mice. Tumours were checked at least four times per week by palpation and inspection. Palpable tumours formed in 10-21 days. The blackest tumours were selected, excised, the tumour nodules were mechanically dispersed in HBSS, then digested with a mixture of deoxyribonuclease (10 μg/ml), collagenase (1 mg/ml), and hyaluronidase (2.5 U/ml) for 15-30 min. A single cell suspension was stored in 20% DMSO and 80% FCS in liquid nitrogen. For *in vitro* passages, B16-F10 cells were cultured in complete DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM β-mercaptoethanol, 2 mM L-glutamine and 200 nM PMA. Only passages 1 and 2 from the liquid nitrogen stock of the B16-F10 cells were used in this study.

mTRP-2 peptide

The antigenic mTRP-2 peptide (VYDFFVWL, TRP-$2_{181\text{-}188}$) (2) was synthesized with a free COOH terminus by Auspep Pty Ltd (Australia). The peptide was >70% pure, as indicated by HPLC, dissolved in DMSO and stored at -70°C.

Construction of viral plasmids and production of replication-defective retroviral particles

The entire *mTRP-2* cDNA was released by double digestion with restriction enzymes Bgl II and Pvu II from plasmid, pcDNA3-clone 3.6 (2) (a gift from Dr. James C. Yang, National Cancer Institute, NIH, Maryland, USA), subcloned into pHR' that had previously been digested with Xho I, blunt ended with Klenow fragment (Promega), then cut with BamH I (compatible with Bgl II). Plasmids, pHR', pHR'CMVLacZ (transfer vector encoding β-galactosidase) and the packaging construct, pCMVΔR8.2, were provided by Dr. Tal Kafri, the Salk Institute for Biological Studies, California.

Replication-defective retroviral particles were generated by transient cotransfection of 293T cells as reported previously (27, 28).

The medium was harvested 48 and 72 hours post-transfection, centrifuged at low speed to remove cell debris and filtered through a 0.45 μm filter. Ultracentrifugation was performed on the filtrate at 50,000 × g for 120 min, and viral particles were resuspended in serum-free RPMI. To obtain titers of the viral stocks, 293 cells were transduced with serially diluted supernatants. For each transduction 8 μg/ml of polybrene (Sigma) was included in the culture medium.

Culture of DCs from mouse bone marrow

Female mice C57BL/6 (H-2b) at 6 week-old were purchased from Animal Resources Centre (Perth, Australia) and held under SPF conditions in the Herston Medical Research Centre (Royal Brisbane Hospital, Australia). Femurs of mice were removed and cut at both ends with scissors. The

marrow was flushed with DC culture medium (DCCM: RPMI-1640, 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B (Fungizone; GIBCO BRL)) using a syringe with a 23G (0.6 mm diameter) needle. The suspension was vortexed to resolve marrow clusters and washed once with DCCM. Approximately 1-1.5 × 10^7 leukocytes were obtained per mouse and seeded at 3 × 10^6 per sterile 100 mm diameter bacteriological petri dish (SARSTEDT, Germany) in 10 ml DCCM containing 200 U/ml rmGM-CSF (Peprotech, Germany). On day 3 another 10 ml DCCM containing 200 U/ml rmGM-CSF was added to the plate for refreshing. On day 6, 10 ml of the culture supernatant was collected, centrifuged (10 min, room temperature, 1,000 rpm, Sigma-203 bench top centrifuge), the pellet was resuspended in 10 ml of fresh 200 U/ml rmGM-CSF DCCM and returned to the original plate to preserve unattached cells. On day 9, non-adherent cells were collected, centrifuged, resuspended in 12 ml of DCCM containing 50 U/ml rmGM-CSF and 500 U/ml tumour necrosis factor α (TNF-α, Peprotech), and then seeded into a fresh 100 mm tissue culture dish (TPP, Switzerland) to mature the DCs. For transduction experiments, 9-day old DCs were incubated with DCCM containing the virus vector, 50 U/ml rmGM-CSF and 500 U/ml TNF-α. FACS analysis of DCs was carried out by staining of the suspension of 1 × 10^5 cells with the following antibodies: FITC-conjugated I-A^b (A_α^b, clone AF6-120.1), FITC-CD86 (B7-2, clone GL1), PE-conjugated CD14 (clone rmC5-3), PE-CD11b (integrin α_m chain, Mac-1 α chain, clone M1/70) and CD11c (integrin α_x chain, clone HL3). FITC-rat IgG2a and PE-hamster IgG (anti-TNP) antibodies were used as isotype controls. All antibodies were from Pharmingen, Germany. Cells were fixed with 1% paraformaldehyde in PBS for FACS analysis.

Allogeneic mixed leukocytes reaction (MLR)

Splenocytes were obtained from BALB/c mice. Briefly, spleen was grated through 70-μm cell strainer (FALCON®) to obtain suspending splenocytes. These cells were then passed through a nylon-wool column to remove APCs. Collected cells were lysed of erythrocytes by resuspending the cells in 17 mM Tris-HCl, pH 7.2, containing 144 mM ammonium chloride and incubated for 1 min at room temperature. Triplicates of 1 × 10^5 splenocytes were seeded into a U-bottom 96-well plate (Nunc, Denmark) with titrated numbers of 7- or 10-day old DCs in the ratio of 1:1280, 1:320, 1:160 and 1:80 DCs to splenocytes. Cultures were pulsed with 1 μCi of ^{3}[H]-methyl-thymidine (Amersham) overnight after 3.5 days. The cells were then harvested onto glassfiber filtermats with Harvester 96® (Tomtec Inc.) and filtermats counted in a 1450 MicroBeta Jet counter (Wallac, Finland).

Lentiviral vector-mediated mTRP-2 gene transfer to DCs

Transduction of DCs was carried out as follows. Non-adherent cells (5 × 10^5) collected from bone marrow-DC culture (day 9) were pelleted and resuspended in 200 μl (approximately 2-3 × 10^7 TDU/ml or multiplicity of infection (MOI) of 10, MOI was derived from *in vitro* transduction of 293 cells) of concentrated vector stock. The following reagents were included in the transducing media; polybrene 4 μg/ml (higher concentration appeared to be detrimental to DCs), 50 U/ml rmGM-CSF and 500 U/ml TNF-α. The mixtures were placed on a suspension mixer (Selby) and rotated gently for 4-5 h. After the incubation the cells were seeded in tissue culture plates with 800 μl of DCCM containing 50 U/ml rmGM-CSF and 500 U/ml TNF-α which were incubated for 48 h before use.

Immunodetection of mTRP-2 expression in transduced DCs

The HR'CmT2 transduced and non-transduced (negative control) DCs were collected and washed with 1× PBS twice before resuspending at 1 × 10^6 cells/ml in 1× PBS. Cell suspension (20 μl) was placed onto the wells of a spot slide and air-dried thoroughly. The cells were fixed by immersing the slide in -20°C acetone for 10 min. After removing the fixative with 1× PBS the cells were coated with 20 μl of undiluted FCS and incubated for 15 min at 37°C in a closed moist chamber to block unspecific binding. The preparation was then washed with 1× PBS several times before incubating with 50 μl 1/50 dilution of αPEP8, a rabbit polyclonal antiserum generated against mTRP-2 (29, 30) for 3 h at 37°C (αPEP8 was a gift from Dr. V. Hearing, NIH, Bethesda, MD, USA). Unbound antibody was removed with 1× PBS and bound primary antibody was detected with 50 μl of anti-rabbit IgG-fluorescein, $F(ab')_2$ fragment (20 μg/ml) for 60 min at 37°C. The cell preparation was then washed 6 times with 1× PBS and redistilled H_2O respectively, and embedded with DAKO® fluorescent mounting medium (DAKO, USA). Fluorescent cells were viewed using an epi-fluorescent microscope (Nikon eclipse E600, Japan) with fluorescein isothiocyanate (FITC) excitation-emission filter (470 nm).

Tumour protection studies

Nine day-old DCs were harvested and transduced as previously described. Peptide-pulsed DCs were included as a control and prepared by loading 11-day-old cells with 10 μg/ml (approx. 1 × 10^{-5} M) of mTRP-2 derived peptide (2 × 10^6 cells in 100 μl DCCM) and incubated at 37°C for 1 h. Before immunisation, non-modified and modified DCs were washed twice in HBSS and 1 × 10^5 cells/100 μl of HBSS were injected subcutaneously (*s.c.*) at the tail base. Ten days after immunisation, 1 × 10^5 trypan blue-excluding B16-F10 cells in 100 μl HBSS were injected *s.c.* in the interscapular area. After tumour challenge, tumour growth was measured every two days with a micro-calliper. Experiments included 6 mice per group and mice that developed tumours > 1 cm in diameter were euthanased and data were recorded in a Kaplan-Meier plot.

Depletion of $CD4^+$ or $CD8^+$ cells in mice administered with transduced DCs

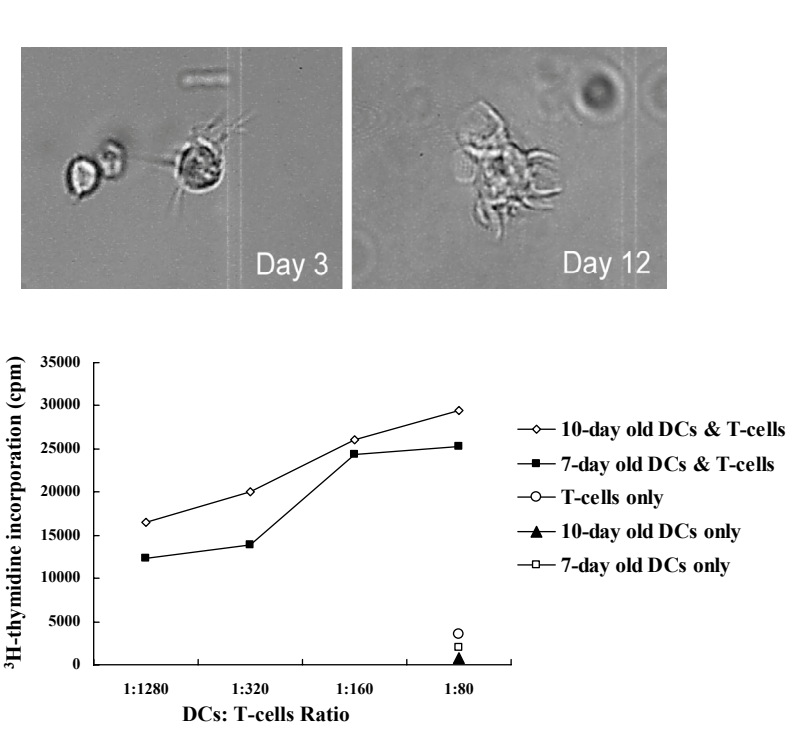

Figure 1. Isolation and functional characterisation of dendritic cells. (A) Photographs of non-adherent cells from the GM-CSF treated BMC culture (400×). (B) Seven and ten-day old DCs (from C57BL/6 mice) were tested for their capacity to stimulate 1×10^5 allogeneic T-cells (from BALB/c) within 3.5 days of co-culture. Radioactive thymidine was added overnight before the cells were harvested for the measurement of ^{3}[H]-thymidine incorporation. The standard deviation of duplicate MLR cultures was below 10%. DCs (1×10^4 cells) and T-cells (1×10^5 cells) by themselves were not proliferating significantly.

This was performed as per the experiment described above with periodic intraperitoneal (*i.p.)* injections of 100 µl anti-CD4 (Mab GK1.5) or anti-CD8 (Mab 2.43) ascitic fluid. Five groups of mice (n = 4) were studied. Group 1 was $CD4^+$ depleted by three *i.p*. injections of 100 µl of anti-CD4 and group 3 $CD8^+$ depleted by injection of anti-CD8 eight days before the immunization. All groups of animals were immunized on day 0. Then on day 1, group 1 was $CD4^+$ depleted again by one *i.p*. injection of anti-CD4, group 2, by three *i.p.* injections of anti-CD4. Group 3 was also $CD8^+$ depleted again by one *i.p.* injection of anti-CD8, group 8, by three *i.p*. injections of anti-CD8. No depletion was peformed on group 5. FACS analysis was performed on all groups of mice on day 9 and tumour challenge on day 10. On day 11, group 2 was $CD4^+$ depleted and group 4 $CD8^+$ depleted.

Depletion was monitored by cytofluorimetry of peripheral lymphocytes isolated from mouse blood. Briefly, each mouse was tail-bled (100 µl) at the designated time to check the completeness of depletion *via* FACS. The collected blood of each group was lysed with Red Blood Cell Lysis Buffer (Sigma) and stained with GK1.5, 2.43 antibodies and subsequently with FITC-conjugated secondary antibody. All of the antibody reagents were generously supplied by Prof. R. Tindle (SASVRC, Royal Children's Hospital, Australia). In each case, more than 98% depletion was observed. Tumour growth was monitored every 2 days and mice that developed tumours > 1 cm in diameter were euthanased and data were recorded in a Kaplan-Meier plot.

Tumour rejection studies in mice bearing established melanomas

Groups of seven C57BL/6 were injected *s.c.* in the interscapular area with 1×10^4 B16-F10 melanoma cells on day 0. Mice were treated with 4 *s.c*. injections of 1×10^5 DCs or DC-HR'CmT2 or PBS (days 4, 8, 12 and 16) at the tail base. After tumour inoculation on day 0, tumour growth was measured every second day with a micro-caliper. Mice that developed tumours > 1 cm in diameter were sacrificed.

Statistical analysis

Statistical analyses of data obtained were performed with the Log-Rank test and Kaplan-Meier Survival Analysis using GraphPad Prism version 3, GraphPad Software, Inc. Comparison of Kaplan-Meier plots was considered statistically significant when the *p* value is less than 0.05.

Results

Culture of DCs and confirmation of function by allogeneic mixed lymphocyte reaction (MLR)

The DC culture was generated from bone marrow cells (BMC). By day 3-5 in culture different cell morphologies were observed. Most easily detected were granulocyte clusters in suspension and macrophages adhering to the bottom of the plate. During this time a very small number of cells with distinctive dendrites started to appear on day 3-5 (Figure 1A). By day 8-10, typically, 70-80% of non-adherent cells showed cytoplasmic process and by day 12 of culture, the unique veil morphology of DCs was evident on the majority of the cells (Figure 1A). Furthermore, FACS analysis of 12-day-old culture confirmed the cell identity, i.e.: high expression of MHC class II molecules ($I\text{-}A^b$), CD86 and

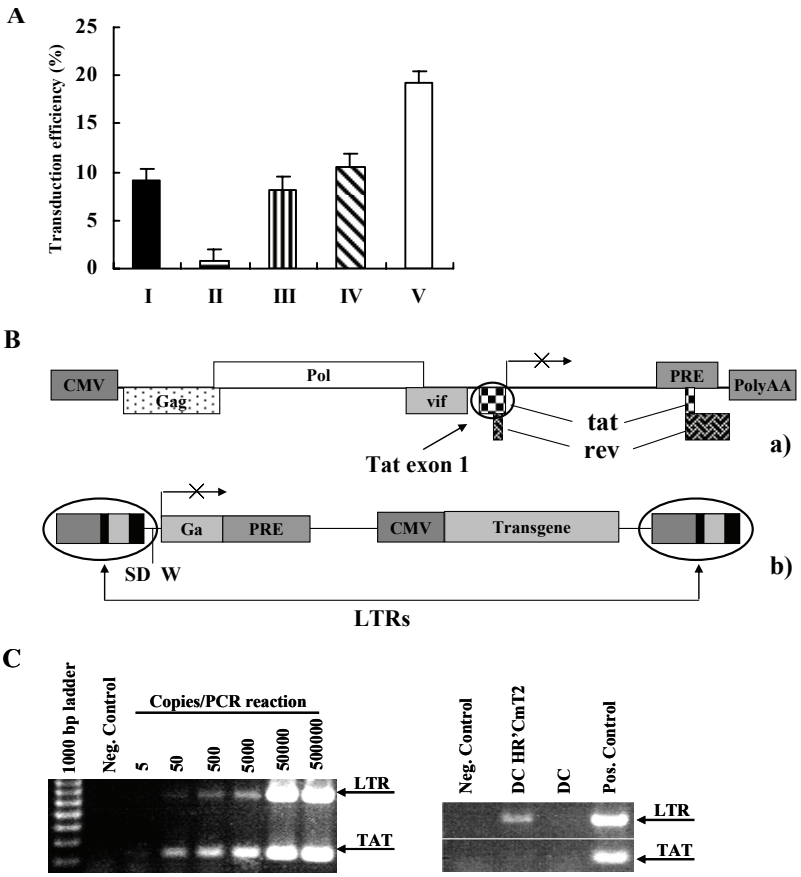

Figure 2. Lentiviral vector-mediated efficient gene transfer and detection of replication-competent viruses. (A) DC transduction efficiency with different conditons. I) MOI 5, 3 cycles of transduction, 2 h each. II) MOI 5, overnight transduction. III) MOI 10, overnight transduction. IV) MOI 10 + 5 mM sodium butyrate. V) MOI 20, overnight transduction. (B) Diagramatic representation of packaging construct and transfer plasmid used in the production of HIV-1 based viral vector. The regions employed in the multiplex PCR are circled. The presence of both amplicons from cellular DNA indicates the unwanted transmission of *tat* and possible recombination events between the two. The sensitivity of this multiplex PCR was performed with serially diluted copy number of the two plasmids. (C) Sensitivity of the "in-house" multiplex PCR. *Tat* amplicon, smaller in size, had better sensitivity than the *LTR* region. It was easily detected at 50 copies/reaction and 5 copies were just able to be detected in 25 μl, 50 cycle PCR. (D) Multiplex PCR for *tat* and *LTR* regions performed on DC-HR'CmT2 resulted only in the amplification of the *LTR* region, indicating that there was an unlikely transmission of the packaging sequences to the target cell.

the DC specific marker (CD11c) were detected (90%), while very few cells displayed the macrophage marker, CD14.

MLR was performed on DCs to assess their ability to stimulate quiescent T cells. MLR is the simplest test of functional capacities of DCs as APCs. Figure 1B showed that bone marrow-derived DCs were functionally active *in vitro* as potent initiators of T-cell proliferation. Comparison of DCs after 7 and 10 days of culture in stimulating allogeneic T cells showed slightly better stimulation by the day 10 DCs. These results suggested the successful generation of DCs from the bone marrow of C57BL/6 mice.

Production of lentiviral vectors-encoding mTRP-2 for TRP-2 gene transfer to DCs

The cDNA fragment encoding mTRP-2, has been shown previously to express the full length antigen under the control of CMV promoter (2) which was subcloned into the HIV-1 transfer vector plasmid, pHR'. The resulting transfer vector was designated pHR'CmT2. The production of the HR'CmT2 lentiviral vectors was carried out as previously reported (27, 28). *In vivo* or *in vitro* are performed routinely on target cell cultures to prove the absence of RCR.

Transduction of DCs was first demonstrated with HIV-1 based vector containing *LacZ* as a marker gene. Staining of DCs exposed to HR'CMVLacZ with X-gal substrate proved successful gene transfer with approximate 20% transduction efficiency using multiplicity of infection (MOI) of 5-20 (Figure 2A). Once the PCR and RT-PCR experiments showed that *mTRP-2* gene transfer *via* the lentiviral vector was successful in the easily transduceable cell lines such as 293 and Hela, transduction was performed on DCs and mTRP-2 production was verified with RT-PCR (Figure 2B).

This test was then performed on genomic DNA of DC-HR'CmT2. As expected only the LTR region was amplified from the transduced cells (Figures 2C and 2D), indicating the presence of proviral transfer vector DNA and the absence of packaging vector sequence.

There were no obvious morphological changes after lentiviral vector-mediated gene transfer.

Induction of protective immunity by adoptive transfer of TRP-2 gene modified DCs.

To induce a protective immunity, a B16-F10 melanoma model was used. In most of the previously reported experiments, antigen-loaded DCs were delivered intravenously (*i.v.)* (10, 31-33), though successes with subcutaneous (*s.c.*) and intraperitoneal (*i.p.*) injections have also been reported (11, 34, 35). DC biodistribution resulting from *i.v.* administration resulted in accumulation in the spleen while *s.c.* injected DCs migrated more to the draining lymph nodes (36).

In this experiment, peptide-pulsed DCs were compared with *mTRP-2* gene-modified DCs. The strongest immune

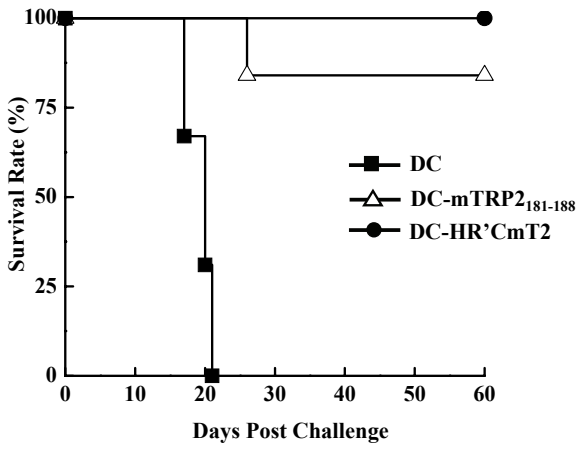

Figure 3. *mTRP-2* gene modified DCs induce protective immunity to melanoma challenge. C57BL/6 mice were vaccinated with DCs, mTRP-2 peptide-pulsed DCs and DC-HR'CmT2. Each mouse was challenged with 1×10^5 of melanoma B16-F10 cells, *s.c*. interscalpular area, 10 days after immunisation. The animals were sacrificed once the tumour was 10 mm in diameter. The mice were monitored till the 60th day post challenge. The values are expressed as percentage of tumour-free mice at the indicated time after challenge. The Log-Rank test gave the following results: DCs *vs* pulsed DC-mTRP-$2_{181\text{-}188}$, $p = 0.0007$; DCs *vs* DC-HR'CmT2, $p = 0.0007$; DC-mTRP-$2_{181\text{-}188}$ *vs* DC-HR'CmT2, $p = 0.3173$.

protection was from *mTRP-2* gene-modified DCs. Peptide-pulsed DCs had some degree of protection. Complete protection and 83% survival rate were observed from DC-HR'CmT2 and peptide-pulsed DCs vaccinated groups respectively. Thus, transduced DCs were more efficient than class I peptide-pulsed DCs (Figure 3) while mice from the control group using unmodified DCs were not protected against B16-F10 challenge, indicating that DCs alone could not induce immunity independently of tumour-specific antigen.

Study of the mechanism of the protection by depletion of $CD8^+$ and $CD4^+$ T cells

To study whether the protective immunity was induced by DC-based vaccination, depletion of the $CD8^+$ and $CD4^+$ were examined and assessed by FACS analysis. Our results showed the depletion of target cells was more than 98% after 3 ascitic fluid injections.

Depletion of $CD4^+$ T cells during the induction phase resulted in termination of antitumour immunity conferred by DC-HR'CmT2, in comparison, the survival rate of DC-HR'CmT2 vaccinated mice was not significantly different from mice in the control group given non-modified DCs ($p = 0.2771$). The results indicated the helper effect of $CD4^+$ T cells. Depletion of $CD4^+$ T cells just prior to tumour challenge did not affect the interactions of DCs and T helper cells and T-cell help for CTL priming was available at the time of vaccination resulting in a survival rate statistically better than DC vaccinated control mice ($p = 0.0069$). However, these results also showed that the immune system without $CD4^+$ cells was not proficient at eradicating tumours. $CD4^+$ T cells activity appeared essential at the time of DC immunisation and tumour establishment, although the function of these cells seemed to be quantitatively more important during the induction phase of tumour immunity (37, 38).

Depletion of $CD8^+$ cells prior to and after vaccination showed that DC-HR'CmT2 immunised mice was effectively protected from tumour challenge and these mice developed notably slower tumour growth ($p = 0.0346$; $p = 0.0344$).

Discussion

mTRP-2 is one of the best known clinically relevant melanoma-associated antigens. Successful transfer of the *mTRP-2* gene into DCs and adoptive transfer of the genemodified DCs could break the immune tolerance and induce host immune response. Lentiviral vectors offer distinctive advantages to achieve this goal as they have effectively transduced dividing and non-dividing cells *in vitro* and *in vivo* with expression of the genes of interest in a sustained fashion in various cell types, such as hematopoietic stem cells, terminally differentiated cells such as neurons, or retinal photoreceptors. The first clinical trial with lentiviral vector (HIV-1 based vector for anti-HIV therapy) has begun.

In this project, we showed that lentivector was an efficient vehicle for mediating foreign gene transfer to DCs. Transduction of DCs was proficiently achieved with HR'CMVLacZ or HR'CmT2 without the requirement of complicated and time-consuming methods which were sometimes employed in DC transduction (18, 19). Furthermore, this vector did not carry with it any viral genes; therefore its use should cause no vulnerability to the DCs. Lentivector-mediated introduction of *mTRP-2* into professional APCs such as DCs will allow prolonged presentation of the antigenic epitopes to the immune system in the context of the many immunostimulatory signals unique to DCs. As DCs have potent T cell-stimulatory capacity, DCs genetically modified to express tumour-associated antigen should be a valuable *ex vivo* approach for immune therapy of tumours.

In vivo adoptive transfer of *mTRP-2* gene modified DCs to a murine melanoma model showed strong and complete protection of the mice from further B16 tumour cell challenge. In comparison, mTRP-2 epitope peptide-pulsed DCs only confers a partial protection (83%). The stronger protection from DC-HR'CmT2 than peptide-pulsed DCs was probably because the transduced DCs were presenting the epitope TRP-$2_{181\text{-}188}$ as well as other yet unidentified class I and particularly class II epitopes. Studies with TRP-$2_{181\text{-}188}$-pulsed DCs have previously shown only partial protection against tumour challenge and the preventative effect appeared to require 2 or more injections (39). However, vaccination of DCs transduced with recombinant adenovirus

encoding the *mTRP-2* gene completely prevented metastatic growth of B16 melanoma cells in mice. This suggested that i) there may be multiple epitopes from mTRP-2 that are essential for effective immunity, ii) the TRP-$2_{181-188}$-MHC class I complex derived from peptide pulsing was not as stable *in vivo* as those derived from transduced cells. The concentration of the peptide has been found to be important in inducing appropriate CTLs. Zeh et al. showed that low concentrations of peptide produced high avidity CTLs, while CTLs generated in the presence of high concentrations of antigenic peptide were of low avidity (40).

$CD4^+$ T cells were important both in the induction phase of the immune response to transduced DCs and of lesser but significant importance in the effector phase. We found that $CD4^+$ T-helper cells were required to induce protective and assist the therapeutic antitumour effect. $CD8^+$ T cells, as expected, were important in both phases. Similar important role for $CD4^+$ T cells was also indicated by Tuting and colleagues, who reported that immunity to B16 melanoma in mice dependent on tumour-specific $CD4^+$ T cells both during the priming phase and at the time of tumour challenge (4).

Further investigations will aim at optimisation of the protocol to develop it as a *via*ble treatment strategy for existing melanoma, such as multiple vaccination regimes, transduction of DCs to present several MAAs concurrently, or the mechanisms of tumour escape. From the data obtained, it can be said that DCs modified to express the MAA, mTRP-2, are beneficial as a prophylactic and therapeutic agent.

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