Lentiviral vectors for induction of self-differentiation and conditional ablation of dendritic cells

M Pincha1, G Salguero1, D Wedekind2, BS Sundarasetty1, A Lin3, N Kasahara4, MH Brugman5, AC Jirmo6, U Modlich5, R Gutzmer7, G Büsche8, A Ganser1 and R Stripecke1

Development of lentiviral vectors (LVs) in the field of immunotherapy and immune regeneration will strongly rely on biosafety of the gene transfer. We demonstrated previously the feasibility of ex vivo genetic programming of mouse bone marrow precursors with LVs encoding granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), which induced autonomous differentiation of long-lived dendritic cells (DCs), referred to as self-differentiated myeloid-derived antigen-presenting-cells reactive against tumors (SMART-DCs). Here, LV biosafety was enhanced by using a DC-restricted and physiological promoter, the major histocompatibility complex (MHC) II promoter, and including co-expression of the herpes simplex virus-thymidine kinase (sr39HSV-TK) conditional suicide gene. Tricistronic vectors co-expressing sr39HSV-TK, GM-CSF and IL-4 transcriptionally regulated by the MHCIi promoter or the ubiquitous cytomegalovirus (CMV) promoter were compared. Despite the different gene transfer effects, such as the kinetics, levels of transgene expression and persistency of integrated vector copies, both vectors induced highly viable SMART-DCs, which persisted for at least 70 days in vivo and could be ablated with the pro-drug Ganciclovir (GCV). SMART-DCs co-expressing the tyrosine-related protein 2 melanoma antigen administered subcutaneously generated antigen-specific, anti-melanoma protective and therapeutic responses in the mouse B16 melanoma model. GCV administration after immunotherapy did not abrogate DC vaccination efficacy. This demonstrates proof-of-principle of genetically programmed DCs that can be ablated pharmacologically.

INTRODUCTION

Dendritic cells (DCs) display a potent ability to stimulate naïve and memory T-cell responses. The past decade has witnessed numerous clinical trials using DCs as immunotherapeutic agents for cancer treatment, in particular melanoma. In spite of the usual correlation between vaccination and immune responses, occurrence of objective clinical responses remained sporadic. One of the proposed issues for upscaling clinical trials is the standardization of ex vivo-cultured DCs, importantly regarding their quality and viability. Hence, practical and consistent technologies shortening the time of ex vivo cell manipulation, although maximizing cell viability and antigen-presentation capabilities are warranted for larger trials.

Several studies have evaluated lentiviral vectors (LVs) for genetic manipulation of ex vivo-grown DCs, demonstrating effective expression of tumor antigens and associated immune responses. In addition to the LV-mediated antigen loading, we explored LV transduction for co-expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) to induce human and mouse DC precursors to self-differentiate. These DCs are heretofore named self-differentiated myeloid-derived antigen-presenting-cells reactive against tumors (SMART-DCs). In mice, SMART-DCs injected one day after LV gene programming showed high viability (>40 days), high bio-distribution to lymph nodes and antigen-specific immune-stimulatory potential. The superior immunotherapeutic potency of SMART-DCs compared with ‘conventional’ DCs was demonstrated in the aggressive B16 melanoma model, transplantable to C57BL/6-immunocompetent mice.

In preparation for clinical translation of the SMART-DC approach, we explored LV designs for improved biosafety. Self-inactivating LVs are currently regarded as a relatively safe vector system: they demonstrated a low genotoxicity profile in a tumor-prone mouse model and have been evaluated in clinical trials involving hematopoietic stem cells with no adverse effects so far. Nevertheless, the concept that ‘physiologic’ promoters in LVs might offer higher safety than constitutive promoters harboring enhancer elements has recently been proposed. As a corollary, in vitro genotoxicity studies of self-inactivating LV expressing the Wiskott–Aldrich syndrome protein under control of its own promoter demonstrated no transforming
potential, which may be a positive scenario for stem cell gene therapy.

We had previously demonstrated the feasibility of inducing mouse BM precursors to self-differentiate into SMART-DCs. Subsequently, we validated the SMART-DCs in their immunotherapeutic potential in a mouse melanoma model with or without ablation with GCV.

RESULTS

Tricistronic vectors induce DC self-differentiation

Here, we compared side-by-side tricistronic LVs containing the CMV promoter or the MHCII promoter and co-expressing HSV-TK, GM-CSF and IL-4 in their capacity to induce mouse bone marrow (BM) precursors to self-differentiate into SMART-DCs. We determined the yield of total viable cells and frequency of transduced BM cells. The lowest cell viability was observed for the MHCII-SMART-DC group, and the viability could be compensated by addition of exogenous recombinant cytokines to the culture. Analyses of the frequencies of viable DCs showed that both conventional and CMV-SMART-DCs underwent a slight loss of viability from days 1 to 3, which recovered to the initial frequency on day 7. For MHCII-SMART-DCs, the viability dropped more substantially from day 1 to 3 (30%), bouncing back on day 7. Addition of rGM-CSF/rIL-4 to MHCII-SMART-DCs culture reduced the loss in viability from days 1 to 3. Altogether, these results demonstrated that for the first week of culture, CMV-SMART-DCs were more viable than MHCII-SMART-DCs, possibly due to the stronger and faster activity of the CMV promoter, leading to higher expression of GM-CSF/IL-4 needed for DC differentiation and survival.

Dendritic cell immunophenotype

The kinetics of SMART-DC differentiation during the first week of culture (days 1, 3 and 7) and at later time points (days 14 and 21) were evaluated by immunophenotypic analyses of characteristic myeloid DC markers (CD11c, MHCII, CD11b). On day 7, the frequencies of SMART-DCs induced by the two different promoters were comparable (around 90%), but the expression of the MHCII marker was consistently higher in MHCII-SMART-DCs than in CMV-SMART-DCs (41–43%) (Figure 2a). On days 14 and 21 of culture, the frequencies of SMART-DCs induced by the two different promoters were comparable (around 90%), but the expression of the MHCII marker was consistently higher in MHCII-SMART-DCs than in CMV-SMART-DCs (41–43%) (Figure 2a). On days 14 and 21 of culture, the frequencies of SMART-DCs induced by the two different promoters were comparable (around 90%), but the expression of the MHCII marker was consistently higher in MHCII-SMART-DCs than in CMV-SMART-DCs (41–43%) (Figure 2a).

Effects of the promoters on the characteristics of SMART-DC differentiated in vitro

The effects of two different promoters on the induction of SMART-DCs were evaluated in two independent experiments performing kinetic analyses on days 7, 14 and 21 after gene transfer. The parameters analyzed were: vector copy number, transgene expression, cell viability and expression of MHCII as an immune-relevant marker. The number of integrated LV copies in the cells was quantified by realtime quantitative polymerase chain reaction. CMV-SMART-DCs and
MHCII-SMART-DCs contained similar LV copy numbers on day 7 (approximately 6–10 copies per genomic DNA of SMART-DCs) (Figure 3a). For CMV-SMART-DC cultures, the numbers of LV copies in the cell population decreased steadily up to day 21, whereas for MHCII-SMART-DC cultures, the number of LV copies increased until day 14, returning to the initial copy number on day 21 (Figure 3a).

The levels of secreted GM-CSF and IL-4 that accumulated in the culture supernatant during the period of 3 weeks were quantified by enzyme-linked immunosorbent assay (ELISA). On day 7,
Figure 2 SMART-DC immunophenotype: (a) flow cytometry analyses of CMV-SMART-DCs versus MHCII-SMART-DCs on days 1, 3, 7, 14 and 21 of culture, resulting in a typical myeloid DC immunophenotype: CD11c<sup>high</sup>, CD11b<sup>+</sup> and MHCII<sup>+</sup>. The forward and side scatter plots show the distribution of the cell populations over time. (b) Histogram analyses of CD11c<sup>+</sup>/MHCII<sup>+</sup>-positive CMV-SMART-DCs and MHCII-SMART-DCs, representing frequency of cells expressing CD80 and CD86 co-stimulatory markers. Representative data from three independent experiments are shown.
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Cytometric analyses of the MHCII marker demonstrated that, throughout the 3-weeks cultures, MHCII-SMART-DCs expressed more persistent levels of MHCII in comparison with CMV-SMART-DCs (Figure 3d), indicating a more enduring DC activation status. The number of recovered viable CMV-SMART-DCs on day 7 of culture was fourfold higher than for MHCII-SMART-DCs, but the decrease of viability for CMV-SMART-DCs was also more accentuated over time (Figure 3e).

SMART-DCs efficiently migrate to adjacent lymph nodes in vivo

We had previously demonstrated by optical imaging and histology analyses that the earlier generation of CMV-SMART-DCs could migrate from the injection site to adjacent lymph nodes and be detectable for a few weeks. To confirm the same effect with this new modality of SMART-DCs co-expressing sr39HSV-TK in addition to GM-CS/IL-4, experiments were conducted in similar settings. For analyses of viability and biodistribution in vivo, the cells were co-transduced with a LV expressing the firefly luciferase (fLUC) gene. On the day after co-transduction, $5 \times 10^5$ cells were injected s.c. on the flanks of C57BL/6 mice. Viable cells emitting bioluminescence (upon catalyses of the substrate luciferin) were detected and quantified on several time points (Figure 4). On day 7, a strong luminescence signal was detectable on the SMART-DC injection sites, and a ramification of the signal spreading toward the region of the inguinal lymph nodes could be clearly observed (Figure 4a). Kinetic analyses of the signal on the injection sites demonstrated high viability of CMV-SMART-DCs on day 7, decreasing on days 14–21 (Figure 4b). In contrast, MHCII-SMART-DCs were more stable during the initial 2 weeks (Figure 4b). This pattern was reproducible for the analyses of the adjacent lymph nodes, which when explanted showed the bioluminescence signal (Figures 4c and d). Using the quantified bioluminescence signal as a parameter to estimate the proportion of migrated cells on day 7, the signal in the lymph node region corresponded to about 5–10% of the signal detectable on the skin (Figures 4a and b). As another analyses strategy to calculate the ratio of ‘donor’ SMART-DCs that reached the adjacent lymph nodes, we used BM of mice expressing the CDS1.1+ hematopoietic marker in the generation of SMART-DCs, which were injected into CD45.2+ recipient mice. Flow cytometry analyses of DCs (CD11c+/MHCII+) infiltrating the lymph nodes revealed their donor origin by expression of CDS1.1. Through these analyses, we observed that 2–4% of the DCs in the lymph nodes were of donor origin (Figure 4e). This frequency was stable for both the cell types, with a decreasing frequency of CMV-SMART-DCs and an increasing frequency of MHCII-SMART-DCs over time (Figure 4e).

Effects of the LV promoter on SMART-DC viability and conditional ablation with GCV

The viability of SMART-DCs and subsequent conditional ablation through the HSV-TK/GCV strategy was initially assessed in vitro by flow cytometry analyses of propidium iodide staining of CD11c+ cells. On days 7 and 21, CMV-SMART-DCs and MHCII-SMART-DCs cultures were equivalent in terms of the frequency of viable CD11c+ cells, which was approximately 50% (Figure 5a). Starting on day 7, GCV was added every 4 days to the medium of parallel cultures until day 21 at a concentration shown to be nontoxic to DCs not expressing TK (data not shown). The cell viability (compared with untreated groups) in both CMV-SMART-DCs and

CMV-SMART-DCs produced more than 20 times higher GM-CSF and IL-4 levels (approximately 40 ng ml⁻¹ accumulated for each) than MHCII-SMART-DCs (GM-CSF: 0.5 ng ml⁻¹; IL-4: 2 ng ml⁻¹) (Figures 3b and c). GM-CSF and IL-4 production by CMV-SMART-DCs peaked on day 14. For MHCII-SMART-DCs, a steady accumulation of GM-CSF and IL-4 was observed during the 21 days of culture (Figures 3b and c).
MHCII-SMART-DCs, cultured in the presence of GCV, dropped to approximately 13–15% on day 21.

To address the longevity of survival and scheduled elimination in vivo, SMART-DCs were marked with fLUC and analyzed by optical luminescence analyses. One day after co-transduction, CMV-SMART-DCs/fLUC or MHCII-SMART-DCs/fLUC were injected s.c. on the flanks of C57BL/6 mice, and the bioluminescence signal was tracked non-invasively (Figure 5c). On day 7, before GCV administration, the bioluminescence signal was readily detectable and comparable for all groups. For this experiment, the kinetic analyses of CMV-SMART-DC viability in vivo demonstrated a slight increase in signal of the bioluminescence vitality marker from weeks 1 to 2, followed by a steady decrease from week 3 onward (Figure 5d). MHCII-SMART-DCs showed a substantial increase in bioluminescence during the initial 3 weeks, decreasing from week 4 onwards, but with remaining vitality detectable for up to 70 days (Figures 5c and d).

At 7 days after SMART-DC administration, GCV was administered i.p. for 5 days into five mice per cohort (Figure 5b). Compared with
the control untreated mice, mice injected with CMV-SMART-DCs or MHCII-SMART-DCs and treated with GCV showed a conspicuous reduction in the bioluminescence signal for all time points taken (Figures 5c and d). The highest ablation effect was observed on day 21, that is, 2 weeks after GCV treatment had been initiated (Figures 5c and d).
Effects of vaccination on antigen-specific CD8+ T-cell response and on lymph nodes

After validating the high viability and migration of CMV- and MHCII-SMART-DCs in vivo, we determined whether these DCs were capable of antigen presentation in the context of an over-expressed bona fide tumor self-antigen. Thus, SMART-DCs were produced by co-transduction with the tricistronic vectors plus a monocistronic vector expressing the tyrosine-related protein 2 (TRP2) melanoma antigen. One day after co-transduction, $1 \times 10^5$ SMART-DCs/TRP2 were injected s.c. into the flanks of C57BL/6 mice. After a prime/boost (on days -10 and day -3, respectively), the splenocytes were collected and re-stimulated in vitro with the peptide TRP2_180-188 SVYDFFVWL, corresponding to a TRP2 MHC class I (H-2Kb)-restricted epitope in the presence of 50 U ml$^{-1}$ IL-2 as previously described (Figure 6a). As a control, an irrelevant OVA peptide was used. The readout for immunological response was detection of CD8+ T cells producing intracellular interferon-gamma (IFN-$\gamma$). The baseline frequency of CD8+ IFN-$\gamma$+ T cells detectable in splenocytes of non-vaccinated mice and autoreactive to TRP2 was approximately 3.5% (the baseline for nonspecific T cells activated with the OVA epitope was approximately 1%) (Figure 6b). Mice vaccinated with CMV-SMART-DCs/TRP2 showed 9% (OVA baseline 1%) of the CD8+ cells-expressing IFN-$\gamma$, whereas for MHCII-SMART-DCs/TRP2-vaccinated mice, the average frequency was highest, approximately 12% (OVA baseline 4%) (Figure 6b). Despite the high-baseline signal, the frequency of CD8+ IFN-$\gamma$+ T cells detectable in splenocytes of vaccinated mice re-stimulated with TRP2 peptide were significantly higher (Figures 6b; representative examples of the flow cytometry analyses for IFN-$\gamma$ responses are shown in Figure 6c). We had previously reported that SMART-DC vaccination induced an enlargement of the adjacent lymph nodes concurrent with higher frequencies of intranodal DC. These effects were reproduced for the novel generation of CMV-SMART-DC and MHCII-SMART-DC vaccines. We observed that the lymph nodes adjacent to the vaccination site for both SMART-DC types were enlarged and contained higher cell numbers as compared with the contralateral side (data not shown). To determine whether the increased cell numbers were correlated with higher frequencies of DCs in the lymph nodes, the cells were stained and analyzed by flow cytometry. A 3- to 4-fold increase in the number of myeloid DCs (CD11c$^+$/CD11b$^+$/MHCII$^+$) was observed in lymph nodes of mice vaccinated with CMV-SMART-DCs or MHCII-SMART-DCs, compared with phosphate-buffered saline (PBS).

**Figure 6** Antigen-specific T-cell responses generated by CMV-SMART-DCs versus MHCII-SMART-DCs in vivo. (a) SMART-DCs ($1 \times 10^5$) co-transduced with LV-CMV or LV-MHCII (5 μg ml$^{-1}$ p24 equivalent) plus LV-TRP2 (5 μg ml$^{-1}$ p24 equivalent) were used for prime/boost vaccination of C57BL/6 mice on days -10 and -3. On day 0, splenocytes and inguinal lymph nodes were collected for analyses. (b) Percentage of CD3+ CD8+ IFN-$\gamma$+ T cells detectable after in vitro priming of splenocytes with the TRP2 peptide or with the OVA control peptide (PBS represents the non-vaccinated mice) ($N=3$). Error bars represent mean±s.d. $P$-values were calculated by paired two-tailed Student's $t$-test. ‘$P<0.05$’; ‘‘$P<0.01$’; ‘‘‘$P<0.001$’, NS, not significant. (c) Representative example of CD3+ CD8+ IFN-$\gamma$+ T cells re-primed in vitro with TRP2 or control peptide OVA. (d) Percentage of cells with myeloid DC (CD11c$^{hi}$/CD11b$^+$/MHCII$^+$) immunophenotype detectable in the inguinal lymph nodes adjacent to the SMART-DC injection sites ($N=2$).
controls (Figure 6d). Having observed that only a small percentage of the DCs in the lymph nodes correspond to SMART-DCs (2–4%, Figure 4e), this indicates a dramatic influx of host DCs to lymph nodes through paracrine effects.

SMART-DCs/TRP2 protect mice against a melanoma lethal challenge

To compare the efficacy of CMV-SMART-DCs and MHCII-SMART-DCs as vaccines in a disease model, we used the highly aggressive, well-characterized B16 melanoma model. B16 is a cell line transplantable into immunocompetent C57BL/6 mice, which has been used extensively in preclinical immunotherapy studies. Recent studies have adopted the use of B16 cells marked with luciferase to follow tumor growth and kinetics by non-invasive in vivo imaging analysis. This system allows confirming the successful engraftment of the s.c. tumor and facilitates quantitative analyses at early time points, until the volume of the tumor becomes measurable (usually 10–14 days after tumor implantation). Hence, in this study we have used the stably transduced B16 cell-line expressing FLuc (B16-FLuc) to monitor tumor growth by optical imaging analyses.

For protective vaccinations, eight mice per cohort were vaccinated s.c. only once with \(1 \times 10^5\) CMV-SMART-DCs/TRP2 or MHCII-SMART-DCs/TRP2 10 days before challenge with \(5 \times 10^5\) B16-FLuc cells (Figure 7a). Antigen loading into the SMART-DC vaccine was performed by co-transduction with LV-TRP2, as described above. After tumor challenge, the mice were analyzed by optical imaging analyses on day 14 to detect the growth of the tumor at an early time point. A single dose of CMV-SMART-DCs/TRP2 was effective in protecting all mice against the lethal dose of B16-FLuc melanoma. In the control group, only 2/8 mice spontaneously rejected tumors (Figures 7b and c). For MHCII-SMART-DCs/TRP2 vaccination, 5/8 mice survived to melanoma challenge, whereas no mice survived in the control groups (Figures 7b and c). Compared with the PBS control group, survival distribution of the vaccination groups calculated by Kaplan–Meier statistical analysis demonstrated significant protection by both groups (for MHCII-SMART-DCs log-rank test \(P=0.0007\); for CMV-SMART-DC log-rank test \(P=0.0024\)) (Figures 7b and c). As an additional comparative study, we conducted protective vaccinations as per the abovementioned protocol, using a single dose of ‘conventional’ DCs/TRP2 (differentiated in vitro with recombinant cytokines for 7 days and transduced with LV-TRP2) or CMV-SMART-DCs/TRP2. CMV-SMART-DCs/TRP2 provided highest number of survivors and protected all the mice from melanoma challenge (100% survival; \(P=0.0026\)) in comparison with ‘conventional’ DCs/TRP2 (60% survival; \(P=0.0403\)) and non-vaccinated controls (Supplementary Figure S1 and Supplementary Table S1). The long-term protective effect of SMART-DC/TRP2 vaccines was tested by re-challenging the mice that had rejected melanoma for long term (120 days). Upon re-challenge of the eight CMV-SMART-DC/TRP2-vaccinated mice with B16-FLuc, three mice rejected tumors (tumors developed in 100% of the control mice). The five mice, which developed tumors, exhibited a drastic delay in tumor growth compared with controls (Figure 7d). For the MHCII-SMART-DC/TRP2 vaccination, three out of five mice survived the re-challenge (Figure 7d).

Immunotherapy of mice with SMART-DCs/TRP2 against a lethal melanoma challenge

We sought to test the efficacy of SMART-DC vaccine in a melanoma immunotherapeutic setting more closely reflecting the clinical scenario, that is, with established tumors. B16-FLuc tumors were implanted on day 0 and four doses of vaccines were injected s.c.: two on the side of the engrafted tumor (days 3 and 9) and two on the contralateral side (days 6 and 12) (Figure 8a). Tumor engraftment and early tumor growth were confirmed by optical imaging analyses performed on day 14 (Supplementary Figure S2). We observed significant slower tumor growth and mortality for both CMV-SMART-DC- and MHCII-SMART-DC-vaccinated mice (Figure 8b). Kaplan–Meier survival analyses demonstrated that both vaccinated cohorts had significantly higher long-term survival than the control group, yet with more significant statistic values for CMV-SMART-DC (\(P=0.0002\)) compared with MHCII-SMART-DC (\(P=0.0013\)) (Figure 8c).

Subsequently, we evaluated whether GCV administration for SMART-DC ablation affected the therapeutic vaccination. We used the same vaccination schedule as presented above, but included i.p. GCV administration for 5 days starting on day 14 after tumor challenge (Figure 9a). Optical imaging analyses on days 3 and 14 post-challenge confirmed comparable tumor engraftment in all mice (Supplementary Figure 3). Tumor measurements and long-term survival analyses confirmed therapeutic effects of both vaccines (CMV-SMART-DC \(P=0.0008\); MHCII-SMART-DC \(P=0.0123\)) (Figures 9b, c and d). GCV treatment of the control mice resulted in faster tumor growth and death, which may be associated with some degree of toxicity of the pro-drug in treated mice. Nevertheless, vaccinated mice demonstrated no significant differences in the therapeutic effects, whether treated with GCV or not.

DISCUSSION

Ex vivo-generated DC vaccines currently applied in the clinic, face inefficient viability, poor migration to the lymph nodes and difficulties in standardization for larger clinical trials. Therefore, ex vivo genetic induction of DC differentiation using engineered LVs can potentially alleviate these problems, if safe LV systems succeed to allow homeostatic differentiation, maintenance and selective ablation of the DCs. Of special interest to DC genetic programming approaches is the use of physiological promoters that timely correlate the expression of transgenes along with the DC differentiation/maturation stages. We have previously shown that LVs containing the MHCII promoter targeted persistent expression of marking genes to MHCII+ cells, particularly DCs in vivo. Nevertheless, development of LVs as direct vaccines for clinical development is still too premature, as very little is known regarding the effects of LVs on biodistribution, pharmacology, toxicology, germ-line transmission and genotoxicity. Thus, we evaluated an ex vivo gene delivery approach, which offers several advantages regarding safety and assessment of quality control criteria for clinical development.

Here, we showed that tricistronic LVs containing the ubiquitous CMV or a 300-base pair MHCII promoter sequence and driving the co-expression of three genes (HSV-TK, GM-CSF and IL-4) in BM precursors effectively induced SMART-DC generation. The tricistronic vectors contained two heterologous 2A elements to avoid homologous recombinations and instabilities of the vector construct (P de Felipe, personal communication). In addition, as the order of the transgenes in the 2A element-containing vector can affect the expression of the individual transgenes, placed the gene for the cytoplasmatic HSV-TK protein upstream of the secreted GM-CSF and IL-4 protein products, which are sorted to the endoplasmic reticulum for secretion.

The comparative in vitro kinetic studies between SMART-DCs programmed with MHCII versus CMV promoter provided some interesting observations. MHCII-SMART-DCs showed a 20-fold lower secretion of GM-CSF and IL-4 compared with CMV-SMART-DCs, but this did not affect their DC identity criteria such as...
morphology and immunophenotype. This may indicate that the cytokine level produced by MHCII-SMART-DC may be at saturating levels to result into effective autocrine production and consumption of the growth factors by the differentiating DCs. As such, the levels of cytokines secreted by MHCII-SMART-DCs increased consistently throughout the culture period. This was correlated with a more persistent number of integrated LV copies in the cell population, and higher purity of MHCIIhigh cells. These results probably reflect a positive feedback loop: as the cells gradually differentiate, activation of the transcriptional machinery for transactivation of the MHCII promoter upregulated the expression of the transgenes, and this served as a positive selection for the transduced cells in culture. Notably, MHCII-SMART-DCs were more stable in vivo than CMV-SMART-DCs. On the other hand, despite producing higher levels of cytokines and reaching DC differentiation earlier, the population of CMV-SMART-DCs showed a steady loss of integrated vector copies in culture correlating with less stable cytokine production over time. A possible explanation is that high cytokine levels lead the DCs to be

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**Figure 7** Protective vaccinations with CMV-SMART-DCs versus MHCII-SMART-DCs followed by melanoma challenge: (a) Schedule of vaccination and challenge. A single vaccination of $1 \times 10^5$ SMART-DCs co-transduced with LV-CMV or LV-MHCII (2 µg/ml -1 p24 equivalent) plus LV-TRP2 (2 µg/ml -1 p24 equivalent) was performed on day -10 and a lethal challenge with $5 \times 10^4$ B16-FLUC was performed on day 0. On day 14, tumor growth was monitored by optical imaging analyses. Long-term survival of tumor-free mice was followed for 4 months (120 days). Long-term survivors were then rechallenged with $5 \times 10^4$ B16-FLUC cells. (b) Optical imaging analyses of tumors on day 14 for the PBS ‘control’ (CTL) and the SMART-DC vaccinated groups. (c) Kaplan–Meier survival curve after first challenge ($N=8$). (d) Kaplan–Meier survival curve after re-challenge of long-term survivors. ($N=8$ for CMV-SMART-DC; $N=5$ for MHCII SMART-DC). *P*-values were calculated by log-rank Mantel–Cox test.
fully 'licensed' and then senesce, along with the fact that secreted cytokines resulting in paracrine effects toward non-transduced cells in the population would be a transitory effect.

Ablation assays with GCV resulted in decreased viability in vitro and in vivo of CMV-SMART-DCs and MHCII-SMART-DCs. Although it is generally established that effective cell killing through HSV-TK/GCV requires DNA synthesis through cell division, exceptions have been reported. GCV-mediated genotoxicity in post-mitotic cells can be partly explained by active DNA repair machinery in non-dividing cells. An example for the effect of HSV-TK/GCV in non-replicating cells was described for primary hepatocytes transduced with LVs expressing HSV-TK and immediately autotransplanted into monkeys. After long-term engraftment of the transduced hepatocytes (which are typically non-replicating), administration of Valganciclovir resulted in effective ablation of the genetically modified cells. Another study reported the effect of mortality caused by the transduction of quiescent hepatocytes upon systemic administration of adenoviral vectors expressing HSV-TK. Non-proliferating thyrocytes expressing HSV-TK could also be ablated by GCV and this effect occurred by influx into the lymph nodes, induced potent antigen-specific CD8+ T-cell activation, and ultimately provided protection and therapeutic benefit to mice against melanoma development. The therapeutic effects were significantly superior for the CMV-SMART-DC immunizations, and GCV administration did not interfere with the immunotherapeutic treatment.

Therapeutic SMART-DC vaccines resulted in significant prolonged survival of all vaccinated mice but, unlike our previous study with SMART-DCs expressing two antigens simultaneously (mTRP2 and hMART-1), mTRP2 expression alone did not result into full tumor eradication in this current study. As the current study is a preclinical evaluation for future clinical developments of TRP2 as a single non-mutated self-antigen that can be explored for immunotherapy of different types of tumors (melanoma, glioma), we purposely excluded the use of hMART-1, as it does not correspond to a bona fide self-antigen in the B16 mouse model, but is a xenogenic antigen leading to potent immunization effect through heteroclitic epitopes. Numerous clinical trials with DC vaccines for melanoma immunotherapy have been performed. A general conclusion for these studies is that, although measurable immune responses against the tumor could be achieved, they eventually failed to produce objective clinical responses in terms of tumor regression or rejection. In the proof-of-concept of SMART-DCs studies shown herein, measurable antigen-specific immune response was directly correlated with therapeutic benefit, in a very aggressive mouse model. We achieved robust protective effects with a single vaccination and moderate but consistent therapeutic effects with four vaccinations. Other than sporadic vitiligo (which indicates a good prognostic for melanoma immune rejection), we did not observe clinical signs of pathologies in mice treated with
SMART-DCs. Preclinical histopathological and toxicological analyses of vaccinated mice maintained at longer periods of time post-vaccination (more than 1 year) are currently ongoing.

The B16 melanoma growth in C57BL/6 mice represents a well-characterized homologous melanoma model (mouse antigens, non-manipulated immune system) and can therefore be considered as relevant for preclinical feasibility and toxicity studies of SMART-DCs. For future clinical development of SMART-DCs, we will use human monocytes as precursors, as this cell population is abundant in the peripheral blood, bypassing the more complex and risky BM draw procedures. Monocytes will be obtained from leukapheresis, transduced \textit{ex vivo} with LVs, and the cells can be administered directly i.d. or cryopreserved. These cells are able to self-differentiate into DCs \textit{in vivo} and are long lived in immunodeficient mice (Stripecke \textit{et al.}, manuscript in preparation). Importantly, insertional mutagenesis risk factors are relatively low in the genetic programming of human monocytes into SMART-DCs, as monocytes are differentiated cells with low replication rate and upon differentiation into DCs they become post-mitotic, drastically reducing their risk of oncogenesis. As the time of monocyte \textit{ex vivo} manipulation is short (1 day), the costs and cell loss are much lower compared with the conventional DC production procedures. TRP2 constitutes an attractive and poorly explored melanoma antigen for clinical development of human SMART-DCs. CTL precursors against TRP2 have been detected in a

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**Figure 9** Therapeutic vaccinations with SMART-DCs and treatment with GCV. (a) schematic representation of therapeutic vaccination. B16-ILUC cells were used for melanoma challenge on day 0 and four doses of $1 \times 10^6$ SMART-DCs co-transduced with LV-CMV or LV-MHCII ($2 \mu g/ml^{-1}$ p24 equivalent) plus LV-TRP2 ($2 \mu g/ml^{-1}$ p24 equivalent) were injected on days 3, 6, 9 and 12. On day 14, mice (N=3) were treated with GCV injected i.p. for 5 days. (b) Kinetics of tumor growth (volume mm$^3$) as an average (N=3) per group for PBS controls and SMART-DC-vaccinated mice with or without GCV administration. Error bars represent mean+s.e.m. (c) Kaplan–Meier long-term survival curve analyses (N=3). (d) Median survival in days and $P$-values. $P$-values were calculated by log-rank Mantel–Cox test.
subset of melanoma patients, indicating that central tolerance to TRP2 is not complete, even during the slow (hence possibly tolerogenic) disease development of human melanoma, which is a different scenario when contrasted to the ‘explosive’ tumor burden resulting of s.c. implanted B16 cells.

DC vaccines still remain an undelivered promise for ultimately curing melanoma, as the immune-evading tumor cells generally swamp the immunotherapeutic responses. Melanoma is an extremely aggressive and highly metastatic type of cancer notoriously resistant to chemo-, radio- and immunotherapy. With recent advances in the understanding that tumor microenvironment being prevalently immunosuppressive, abrogating the immune-mediated tumor attack, the use of agents, which overcome suppression have been combined with immunotherapy. The most promising candidate showing clinical benefit is an antibody blocking cytotoxic T-lymophocyte-associated antigen-4 (CTLA-4), which is a key inhibitory regulator of immune responses. CTLA-4 receptor is expressed on T cells and binds to its ligand (B7-2 or CD86) present on APCs, inhibiting T-cell responses triggered by CD28. Antibodies blocking the interaction of co-stimulatory molecules with CTLA-4 can lead to a sustained immune response. A recent study by Curran et al. has demonstrated the effective combination of CTLA-4 and programmed death-1 (PD-1) ligand (B7-2 or CD86) present on APCs, inhibiting T-cell responses triggered by CD28. Antibodies blocking the interaction of co-stimulatory molecules with CTLA-4 can lead to a sustained immune response. A recent study by Curran et al. has demonstrated the effective combination of CTLA-4 and programmed death-1 (PD-1) ligand (B7-2 or CD86) present on APCs, inhibiting T-cell responses triggered by CD28. Antibodies blocking the interaction of co-stimulatory molecules with CTLA-4 can lead to a sustained immune response. A recent study by Curran et al. has demonstrated the effective combination of CTLA-4 and programmed death-1 (PD-1) ligand (B7-2 or CD86) present on APCs, inhibiting T-cell responses triggered by CD28. Antibodies blocking the interaction of co-stimulatory molecules with CTLA-4 can lead to a sustained immune response.

**Materials and Methods**

**Construction of vectors**

The self-inactivating LV backbone vector and the monocistronic vectors expressing FLuc (RRL-cPPT-CMV-FLuc) and tyrosine-related protein 2 (RRL-cPPT-CMV-TRP2) were previously described. Construction of LV containing 2A elements was performed by overlapping PCR essentially as previously described. A bicistronic vector (RRL-cPPT-CMV-mGMSF-P2A-mIL4) containing mGM-CSF and mIL-4 separated by a 2A element was constructed by overlapping PCR. Primers were used to generate intersplicing P2A: P2A/IL-4 forward 5'-GCCGGCACTCTCTCTGTGTAAAGAGCGAAGAGAcTTACAAAGCCCGGTCCCGTATGAGGTC-3' and P2A/CM-CSF reverse 5'-GACGTGGAAGAAAACCCCGGTCCTATGGGTCTCAACCCCCAGC-3'. The cDNA encoding s39HSV-TK, a mutant form of HSV-TK with enhanced affinity to its substrate was kindly provided by Prof. Li, Wu (UCLA). The final tricistronic vector was constructed by overlapping PCR with the bicistronic (mGM-CSF-P2A-mIL4) and the (s39HSV-TK) template containing vectors. Primers used to generate intersplicing T2A were: T2A/IL-4 forward 5'-TGGGCAGAGATTCTCTTCCAGTACACGGGATCTCATGCGG-3' and T2A/HSV-TK reverse 5'-GGCTGGGAAAGATTGGGAGGCTAAGGATCCCAGGGAGGGGAGG-3'. The phenotypic analysis of BM-derived DCs was performed exactly as described. LVs were concentrated by ultracentrifugation, typically providing titers of >10^8 transduction units ml^-1. Lentiviral titer was determined by assessing viral p24 antigen concentration by ELISA (Cell Bioslabs, San Diego, CA, USA), and hereafter expressed as μg of p24 equivalent units per ml. A measure of 1 μg p24 equivalent per ml corresponds to approximately 1–5×10^5TU ml^-1 using 293T cells as a reference line.

**Ex vivo generation of genetically programmed DCs**

Bone marrow was aseptically flushed from femurs of C57BL/6 mice, washed and incubated in 10 cm diameter plastic dishes with RPMI supplemented with 10% FBS overnight. Non-adherent cells were plated at density of 5×10^5 cells per well of six-well plates in RPMI with 10% FBS in the presence of recombinant mouse GM-CSF and mouse IL-4 (50 ng ml^-1) each; R&D Systems, Wiesbaden, Germany) for 8 h before transduction. Cells were transduced on six-well plates in the presence of 2–5 μg p24 equivalent per ml of virus plus Protamine Sulfate (5 μg ml^-1; Valeant, Dusseldorf, Germany) for 16 h at 37 °C.

**Determination of vector copy number by RT-Q-PCR**

DNA was isolated using Qiagen Qiaamp Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s guidelines. DNA concentrations were determined on a Nanodrop ND-1000 (Peqlab, Erlangen, Germany) after which 10–100 ng DNA was used in triplicates in multiplexed qPCR using primers for FliK as endogenous DNA control (FliK_forward: 5'-GGTTCAAAATGCTACCGTATTCCCTT-3', FliK_reverse: 5'-CTTGGCCAGACTCGATCCCGT-3' and FliK_probe: 5'-FAM-GTGACCATCTGGCCATTCA-3') and primers for the PRE element in the vector (PRE_forward: 5'-GAGGATTTGTGCCGCGTGTGTG-3', PRE_reverse: 5'-TGGAGACCATTGCCCATTTTTTTTG-3' and PRE_probe: 5'-Yakima Yellow 5'-CTGTGTTTGGCTGAGGACGAC-3' – BHQ, all primers from Eurogentec, Seraing, Belgium). Primers were used at a final concentration of 330 nM and probes were used at a final concentration of 107 nM using Eurogentec qPCR mastermix (Eurogentec) in a total volume of 15 μl, using the following thermal profile: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s. All RT-Q-PCR experiments were performed using a StepOnePlus thermocycler (Applied Biosystems, Darmstadt, Germany). Quantitative PCR data was evaluated using efficiency corrected relative quantification using a cell clone with known LV copy number as the reference sample.

**Flow cytometry analyses of surface antigens and viability stainings**

The phenotype of BM-derived DCs was analyzed as previously described. Briefly, cells were detached from the wells by incubation in PBS (without Mg2+ and Ca2+) for 30 min at 37 °C in 5% CO2 incubator. After incubation, cells were resuspended and washed once with PBS and then incubated with mouse IgG (50 μg ml^-1) on ice for 15 min before further staining with the corresponding monoclonal antibodies. Immunostaining was performed with commercially available fluorescent-immunconjugated monoclonal antibodies against CD11c phycoerythrin conjugated, CD11b FITC conjugated, CD80 FITC conjugated.
conjugated, CD86 FITC conjugated, MHC-II APC conjugated, with respective isotype controls (BD Pharmingen, Heidelberg, Germany). At 30 min after incubation with the monoclonal antibodies, cells were resuspended in 100 µl of 1% paraformaldehyde for fixation. For viability staining, cells were stained on ice with 5 µl of 1% paraformaldehyde for 5 min. After staining with Annexin V conjugated with APC (eBiosciences, Frankfurt, Germany) for 15 min in dark at room temperature. Immediately before flow cytometry analyses, the cells were stained with 7AAD on ice according to manufacturer’s instructions (Beckman Coulter, Krefeld, Germany). For each set of analyses, 1 x 10^6 cells were acquired. To establish background for fluorescence and to set gates for data acquisition, cells stained with isotype antibodies were used as baseline reference. Care was taken to analyze cells that were in the viable white blood cell gate as indicated by forward and side-scatter characteristics. Cells were further analyzed using a FACSCompBiter cuymeter (BD Biosciences, Heidelberg, Germany) and acquisition and analyses were done using Cell Quest software (BD Biosciences) or Summit software (Summit V5, Beckman Coulter, Brea, CA, USA).

Ablation assays with GCC in vitro
GCC (Sigma-Aldrich St Louis, MO, USA) solution was added at a concentration of 33 µM to the cultures of SMART-DCs on day 7 and continued every 4 days until day 21. Cells were collected on days 7, 14 and 21 for analyses by flow cytometry. After immunostaining with CD11c-FTTC, propidium iodide (BD Biosciences) was added and the cells were kept at 4°C for 5 min before analyses. Flow cytometry was performed with a FACSCompBiter flow cytometry apparatus. For each set of analyses, 1 x 10^6 cells were acquired.

Ablation assays with GCC in vivo
SMART-DCs (5 x 10^6) co-transduced with LV-fLUC (5 µg/ml-1 p24 equivalent) were injected i.p on the right flanks (on day 0). Control mice were injected with PBS. Pharmacological grade Cymevene/GCV (5 mg/kg-1) (Recordati, Ulm, Germany) was injected i.p. for 5 days starting on day 7 post-vaccination and mice were killed on day 70.

DC migration analyses in vivo
SMART-DCs (5 x 10^6) generated from BM cells of CD45.1 mice and co-transduced with LV-fLUC (5 µg/ml-1 p24 equivalent) were injected s.c. on the right flanks (on day 0). Control mice were injected with PBS. The draining inguinal lymph nodes adjacent to the injection sites were collected for analyses on days 7, 14 and 21 post-vaccination. Single-cell suspensions were prepared to perform flow cytometry analyses. Cells were blocked with mouse IgG (50 µg/ml-1) on ice for 15 min before staining with the corresponding monoclonal antibodies. Immunostaining was performed with commercially available monoclonal antibodies against CD45.1 phycoerythrin conjugated (eBiosciences), CD11c FITC conjugated, MHC-II APC conjugated (BD Pharmingen) and using respective isotype controls for gating. At 30 min after incubation with the antibodies, cells were resuspended in 100 µl of 1% paraformaldehyde for fixation. Cells (1 x 10^6) were acquired for each set of analyses.

Analyses of CD8+ T-cell responses against TRP2
For intracellular IFN-γ staining, splenocytes were cultured for 6 h in 96-well plates at a concentration of 1 x 10^6 cells per well in 0.2 ml of complete medium with 1 µl/ml-1 brefeldin A (GolgiPlug; BD Biosciences) and human IL-2 (50 U/ml-1). The cells were restimulated with a TRP2 peptide (TRP2: 180–188, SVYDFFVWL) or with a control peptide (OVA: SIINFEKL) (Thermopep, Oxford, UK) at 5 µg/ml-1. The cells were spun down and surface antigens were stained in PBS, supplemented with 2% FBS with FITC-conjugated monoclonal antibodies for CD8a and APC-conjugated monoclonal antibodies for CD3 (BD Biosciences). After washing off the unbound antibody, cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit (BD) according to manufacturer’s instructions. For intracellular IFN-γ staining, the phycoerythrin-conjugated monoclonal rat anti-mouse IFN-γ Ab (clone XMG1.2; BD Pharmingen) or its isotype control Ab (rat IgG1) was used. Flow cytometry was performed with a FACSCompBiter flow cytometry apparatus. For each set of analyses, 5 x 10^6 cells were acquired. To establish background for fluorescence and to set gates for data acquisition, cells stained with isotype antibodies were used as baseline reference. Care was taken to analyze cells that were in the viable white blood cell gate as indicated by forward and side scatter characteristics.

Vaccinations, melanoma challenge and GCV treatment
All cell suspensions diluted in PBS were injected in volumes of 100 µl s.c. on the hind flanks of mice with a 27-gauge needle. For antigen loading, SMART-DCs were co-transduced with LV-TRP2. Mice were always immunized with 1 x 10^5 SMART DCs and challenged with 5 x 10^6 B16-FLUC cells following different schemes indicated for each experiment type. Treatment with GCV (5 mg/kg-1), i.p. started on day 14 and was performed daily for 5 days for protocol of therapeutic vaccinations combined with SMART-DC ablation. Tumor growth was measured every 2 days with a digital microcaliper (Mituyo, Nuees, Germany). Mice that developed tumors > 1.5 cm in diameter or ulcerations were killed according to the regulations of the Hannover Medical School. Mice that did not develop tumors were maintained in observation for at least 3 months after challenge. For statistical analyses of tumor challenge studies, probability of disease-free survival was estimated using Kaplan–Meier’s method; disease-free survival was plotted and compared using log-rank test. All tests were two-sided and P<0.05 was considered significant. GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis.

In vivo bioluminescence imaging analyses
Mice were anesthetized with ketamine (100 mg/kg-1 i.p.) and xylazine (10 mg/kg-1 i.p.), and an aqueous solution of d-Luciferin (150 mg/kg-1 i.p.) was injected 5 min before imaging. Animals were placed into the light chamber of the CCD camera (IVIS, Caliper Life Sciences, Mainz, Germany), and grayscale body surface reference images (digital photograph) were taken under weak illumination. After switching off the light source, photons emitted from luciferase-expressing cells within the animal body and transmitted through the tissue were quantified over a defined period of time ranging up to 5 min using the software program ‘Living Image’ (Caliper Life Sciences) as an overlay on Igor (WaveMetrics, Seattle, WA, USA). Bioluminescence was then quantified in units of photons per second per centimeter square per steradian.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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