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Preclinical evaluation of NF-κB-triggered dendritic cells expressing the viral oncogenic driver of Merkel cell carcinoma for therapeutic vaccination

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Abstract

Background: Merkel cell carcinoma (MCC) is a rare but very aggressive skin tumor that develops after integration of a truncated form of the large T-antigen (truncLT) of the Merkel cell polyomavirus (MCV) into the host's genome. Therapeutic vaccination with dendritic cells (DCs) loaded with tumor antigens is an active form of immunotherapy, which intends to direct the immune system towards tumors which express the respective vaccination antigens.

Methods: Cytokine-matured monocyte-derived DCs of healthy donors and MCC patients were electroporated with mRNA encoding the truncLT. To permit major histocompatibility complex (MHC) class II next to class I presentation, we used an RNA construct in which the antigen was fused to a DCLamp sequence in addition to the unmodified antigen. To further improve their immunogenicity, the DCs were additionally activated by co-transfection with the constitutively active nuclear factor (NF)-κB activator caIKK. These DCs were used to stimulate autologous $CD8^+$ T-cells or a mixture of $CD4^+$ and $CD8^+$ T-cells. Then the percentage of T-cells, specific for the truncLT, was quantified by interferon (IFN)γ ELISpot assays.

Results: Both the truncLT and its DCLamp-fusion were detected within the DCs by flow cytometry, albeit the latter required blocking of the proteasome. The transfection with caIKK upregulated maturation markers and induced cytokine production. After 2–3 rounds of stimulation, the T-cells from 11 out of 13 healthy donors recognized the antigen. DCs without caIKK appeared in comparison less potent in inducing such responses. When using cells derived from MCC patients, we could induce responses for 3 out of 5 patients; however, here the caIKK-transfected DCs did not display their superiority.

Conclusion: These results show that optimized DCs are able to induce MCV-antigen-specific T-cell responses. Therapeutic vaccination with such transfected DCs could direct the immune system against MCC.

Keywords: adoptive cellular immunotherapy, dendritic cells, large T-antigen, Merkel cell carcinoma, polyomavirus

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Introduction

Merkel cell carcinoma (MCC)[1] is a rare but highly aggressive type of skin cancer with increasing incidence.[2,3] In 2008 the MCC-responsible virus was discovered and termed Merkel cell polyomavirus (MCV).[3] This virus is ubiquitous in the human population and seroprevalences around 75% are reported.[4–6] It is usually non-oncogenic, but under

certain circumstances, the viral genome can integrate into the host cells' genome and expresses a mutated and thus truncated form of one of its proteins, termed the large T-antigen (truncLT), while the expression of the other viral proteins is switched off.[7] How this is initiated, is yet not fully understood, but UV irradiation, immune suppression, and high age are associated with this process.[8]

The actual incidence of this cutaneous cancer is 2–8 cases per million people per year in Europe and the USA[2] and 16 cases per million people in Australia.[9] The incidence rate has been constantly increasing since the 1980s.[3,10] MCC is characterized by a high mortality of 46%.[11] Satellite and lymphatic metastases develop rapidly, accompanied by a reduction of the survival rates to below 20%.[11–13] Apart from surgical excision, no effective therapy existed until recently. Radio- and chemotherapy are usually only performed with a palliative intention.[14] However, these interventions did not improve the overall survival for stage III MCC patients.[15] In 2015 this kind of treatment in the context of clinical trials was still considered as standard of care for disseminated MCC.[16] New hope has emerged, as current trials with checkpoint blockade antibodies (reviewed by Vandeven and Nghiem[17]) have shown promising results. This new type of immunotherapeutic antibody targets inhibitory receptors on T-cells and their ligands in order to enhance anti-tumor immune responses.[18] The observed clinical successes indicated that immunotherapy is suitable to target MCC and will probably become the new standard of care,[19] but despite a high response rate, still not every patient responded to the new drugs. Therefore, it is necessary that additional treatment options or new combinations are evaluated.

One approach is to induce tumor-specific T-cells by vaccination with dendritic cells (DCs). DCs are an ideal tool for immunotherapy, because they are able to process and present tumor-derived antigens, they prime, expand, and control-antigen-specific T-cells, and guide their differentiation as nature's adjuvant.[20] These cells were already used in many clinical trials,[21] but they are subject to further optimization.

Artificial activation of the DCs by mRNA-transfection with factors related to NF-κB activation resulted in improved immunogenicity.[22–24] We could show that cytokine-matured DCs, which were transfected with constitutively active IκB kinase β (caIKK), displayed functions associated with better immunogenicity and memory induction, like for example, interleukin (IL)-12p70 secretion.[25]

For DC vaccines, a suitable target antigen is obviously of great importance. Such an antigen should be exclusively expressed in the malignant tissue, and ideally not be germline-encoded to avoid thymic tolerance. A certain protein size would be beneficial to facilitate enough T-cell epitopes within the antigen, and ideally such a protein should contribute to the malignant phenotype of the cancer cells. The truncLT combines these features making it an intriguing cancer vaccination antigen.

In this study, we examined the immunogenicity of truncLT-transfected DCs for immunotherapy and showed the induction of antigen-specific responses in $CD8^+$ or $CD4^+$ and $CD8^+$ T-cells from healthy donors and MCC patients.

Material and methods

Any sequences, constructs, and raw data are available on request from the corresponding author.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were purified from peripheral blood, obtained from healthy donors or MCC patients following informed consent and approval by the institutional review board (Ethikkommision der Friedrich-Alexander- Universität Erlangen-Nürnberg, Erlangen, Germany: Ref. no. 4158 and 4603, respectively) as described before.[26] For the described priming experiments with healthy donor blood, between 400–500 ml peripheral blood was used, divided on 2 blood draws with a 1 week interval. Blood draws from patients were kept lower (usually 60 ml in total) to avoid harm to the elderly and diseased patients. Blood was stored for less than 3 h in the presence of heparin or ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Steinheim, Germany) and subjected to density centrifugation with lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Cells were washed 3 times in Dulbecco's phosphate-buffered saline (DPBS; Lonza, Verviers, Belgium) containing 1 mM EDTA at 282 g for 15 min, 189 g for 10 min and 114 g for 12 min at 4°C. An

additional washing step with RPMI 1640 (Lonza) at 149 g for 12 min was performed and cells were counted with a Neubauer hemocytometer and trypan blue staining (Sigma-Aldrich) before the cells were further processed. Approximately 0.8 to 1.5 $\times$ 10^6 cells could be generated from 1 ml of blood.

Generation and maturation of DCs

The monocytes were isolated from the PBMCs by plastic adherence. A total of 3–4 $\times$ 10^7 PBMCs per 10 cm tissue culture dish (Falcon, Corning GmbH, Kaiserslautern, Germany) were incubated in 10 ml DC medium for 1 h at 37°C. DC medium consisted of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% heat-inactivated non-autologous human plasma from individual donors (Transfusionsmedizin, Universitätsklinikum Erlangen, Erlangen, Germany) or heat-inactivated pooled human serum (Sigma-Aldrich), 2 mM L-glutamine (Lonza), and 20 mg/l gentamycin (Lonza). The non-adherent fraction (NAF) was removed by rinsing with RPMI 1640 and used for T-cell isolation. Approximately 1–2 $\times$ 10^7 non-adherent cells were obtained from each dish. The adherent monocytes were differentiated to immature DCs over 6–7 d in DC medium supplemented with 800 IU/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Miltenyi, Bergisch Gladbach, Germany), and 250 IU/ml IL-4 (Miltenyi) on days 1, 3, and 5. On day 6 or 7 the DCs were matured by adding a cytokine cocktail of 200 IU/ml IL-1β (CellGenix, Freiburg, Germany), 1000 IU/ml IL-6 (CellGenix), 200 U/ml tumor necrosis factor (TNF) (Peprotech, Hamburg, Germany), and 1 μg/ml prostaglandin E_2 (PGE_2) (Pfizer, Zurich, Switzerland) for another 24 h. One culture dish usually yielded between 1–2.5 $\times$ 10^6 cytokine-matured DCs (cmDCs).

Isolation of T-cells

$CD4^+$ and $CD8^+$ T-cells were isolated from fresh and cryoconserved NAF, using anti-CD4 or anti-CD8 MACS-beads (Miltenyi) respectively, according to the manufacturer's instructions. The yield was between 5–10% for $CD8^+$ and 10–20% for $CD4^+$ T-cells. The isolated T-cells were cultured overnight at a concentration of 1–2 $\times$ 10^6 cells/ml in T-cell medium consisting of RPMI 1640, 10% heat-inactivated human serum (Sigma-Aldrich), 2 mM L-glutamine, 20 mg/l gentamycin, 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES; PAA Labortechnik, Pasching, Austria), 1 mM sodium pyruvate (PAA), and 1% MEM non-essential amino acids (aa) (100×, PAA), supplemented with 20 U/ml IL-7 (Peprotech) for $CD8^+$ T-cells and additionally with 5 ng/ml IL-15 (R&D systems, Wiesbaden-Nordenstadt, Germany) for $CD4^+$ T-cells.

Cryoconservation

DCs and NAFs were cryopreserved by resuspending the cells in cold human serum albumin solution (20%, Sigma-Aldrich) at a maximum concentration of 5 $\times$ 10^6 cells/ml (DCs) or 5 $\times$ 10^7 cells/ml (NAF). An equal volume of cryopreservation medium was added, consisting of 55% human serum albumin solution (20%, Sigma-Aldrich), 20% dimethyl sulfoxide (Sigma-Aldrich) and 25% glucose monohydrate solution (40%, Fresenius Kabi, Bad Homburg, Germany). Cells were then frozen at a speed of −1°C/min in a cryopreservation container (Nalgene, Rochester, NY, USA) to −80°C. Thawing was performed by mixing the cells with room temperature RPMI 1640, washing them in at least 10 ml RPMI 1640, and transferring them to a cell culture dish containing DC medium, supplemented with GM-CSF (800 IU/ml) and IL-4 (250 IU/ml) or T-cell medium, supplemented with IL-7. The cells were allowed to rest at 37°C for 1 h before further processing.

In vitro *transcription of RNA*

RNA was generated by *in vitro* transcription using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Life Technologies, Carlsbad, CA, USA) and purified with an RNeasy Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The trLT construct consisted of the aa 1–259 of the MCV large T-antigen fused to a myc-tag sequence. The trLT-DCL construct consisted of the Lamp1 signaling peptide (aa 1–29) preceding the aa 1–246 of the MCV large T-antigen fused to the human DCLamp sequence[27] and a myc-tag sequence. Codon-optimized templates were generated by GeneArt (ThermoFisher Scientific, Schwerte, Germany) and cloned into the pGEM4Z64A RNA production vector.[28] The caIKK construct corresponds to caIKKβ described previously.[25] The control-DCL-RNA consisted of an irrelevant tumor antigen (mutated BRAF and GNAQ), also framed by the Lamp1 signaling peptide and the DCLamp and myc-tag sequence. The complete nucleotide

sequences of all production vectors are available upon request.

RNA electroporation of DCs and T-cells

RNA electroporation (EP) was performed as described.[29] Centrifugation of DCs and T-cells was always performed for 10 min at 22°C and 149 g or 233 g, respectively. DCs were transfected with the RNA amounts indicated in the particular experiment. Prestimulated T-cells were electroporated[26] without RNA, 50 or 150 µg/ml trLT-RNA, 50 or 150 µg/ml trLT-DCL-RNA or 150 µg/ml of the control-DCL-RNA. For electroporation, cells were harvested in RPMI 1640, washed once in OptiMEM without phenol-red (Invitrogen, Karlsruhe, Germany), and then resuspended in OptiMEM with a maximal concentration of 6×10^7 DCs/ml or 12×10^7 T-cells/ml (all at room temperature). Electroporation was performed in 4 mm cuvettes (biolabproducts GmbH, Bebensee, Germany) with a Genepulser Xcell machine (Bio-Rad, Munich, Germany). The conditions were: square-wave pulse, 500 V, and 1 ms for DCs or 5 ms for T-cells, respectively.[29]

After transfection, DCs were rested at 37°C for 4 h in DC medium supplemented with GM-CSF (800 IU/ml) and IL-4 (250 IU/ml), before using them for T-cell expansion or cryoconservation. Transfected T-cells were rested in T-cell medium for 1 h before being used for further experiments. The survival rate of the DCs was around 75% and over 50% when combined with cryoconservation. The survival rate of the T-cells was between 60–80%.

Expansion of antigen-specific T-cells

Electroporated DCs were co-incubated with autologous T-cells, either pure $CD8^+$ T-cells or a 1:1 mixture of $CD4^+$ and $CD8^+$ T-cells, with 2×10^6 T-cells and 2×10^5 DCs in 2 ml T-cell medium supplemented with IL-7 for 1 week. Excess DCs were cryoconserved for restimulation. On the 2nd and the 4th day, 1000 IU/ml IL-2 and 10 ng/ml IL-7 were added and an additional 5 ng/ml IL-15, when $CD4^+$ T-cells were present in the culture. After 1 week, the T-cells were harvested and used for the next round of expansion or for the read-out. For healthy donors, the 2nd stimulation was also performed with fresh, electroporated DCs. This *in vitro* assay uses only human autologous primary cells and hence can emulate the interaction between the DCs and the T-cells, but of course the situation within a living organism is much more complex and the involvement of other cell types is not covered.

Flow cytometric analysis of intracellular trLT-construct expression

For intracellular detection of the introduced trLT and trLT-DCL, the electroporated DCs were treated with 0.5 µm bortezomib or were left untreated. At 4 h after electroporation the DCs were vortexed and fixed with 2% formaldehyde for 10 min at room temperature or overnight at 4°C. After washing cells once with FACS buffer (DPBS with 1% fetal bovine serum (FBS) and 0.2% sodium azide), they were incubated in chilled (−20°C) methanol (⩾99.9%) for 10 min. Afterwards, cells were washed twice with FACS buffer and then stained with the anti-myc-tag Alexa®488 mouse mAb (clone 9B11, NEB, Frankfurt am Main, Germany) in the dark. Immunofluorescence was detected using a FACScan cytofluorometer equipped with CellQuest software [Becton Dickinson (BD), Heidelberg, Germany]. Living cells were gated by forward and sideward scatter. Of note, is that this method only shows the amount of antigen within the cells, but does not show its turnover or the processing and presentation.

Flow cytometric analysis of surface marker expression on DCs

Mature DCs were electroporated without or with IKKβ-RNA as described above and harvested 24 h, 48 h, or 72 h after transfection. Cells were stained at 4°C in FACS buffer for 30 min with the following antibodies: anti-CD40-FITC (BD), anti-CD40-PE (BD), anti-CD25-FITC (Cymbus Technologies, Southampton, Hampshire, United Kingdom or BD), anti-CD25-PE (BD), anti-CD70-PE (BD), anti-OX40L-PE (BD), anti-CD80-FITC (BD), anti-CD83-PE (Miltenyi), anti-CCR7-FITC (R&D Systems), anti-CD86-FITC (Cymbus Technologies), anti-CD86-PE (Miltenyi, BD), and anti-PD-L1-PE (eBioscience) and with matched isotype controls: IgG1-FITC (BD, Miltenyi), IgG1-PE (Miltenyi), IgG2a-FITC (BD), IgG3-PE (eBioscience). The cells were then washed once with FACS buffer and were taken up in FACS buffer or a mixture of equal amounts of FACS-Fix (DPBS with 2% formaldehyde) and FACS buffer. Afterwards, the immunofluorescence was determined using a FACScan cytofluorometer equipped with Cell

Quest software (BD). The DCs were discriminated based on their size and granularity by gating in the forward and side scatter channels. The specific mean fluorescence intensities (specific MFI) were calculated by subtraction of the background mean fluorescence intensity obtained with the isotype control antibodies. All values were set in relation to the 24 h control condition to calculate the fold induction.

Cytokine analyses

Matured DCs were electroporated without or with caIKK-RNA as described above. The cytokine concentrations in the supernatant of the cells were analyzed 24 h after EP with a human inflammatory Cytometric Bead Array (CBA) from BD according to the manufacturer's instructions. Absolute cytokine concentrations were determined from standard curves using the Excel software (Microsoft, USA). Negative values were set to 0.

Interferon γ-ELISpot assays

Interferon (IFN)γ-ELISpots were performed as described before.[26] Anti-IFNγ-coated 96-well ELISpot plates (Mabtech, Hamburg, Germany) were washed once with DPBS (Lonza) and blocked for 1 h at 37°C with ELISpot medium, consisting of RPMI 1640 with 5 % heat-inactivated human serum, 2 mM L-glutamine, 20 mg/l gentamycin, and 10 mM HEPES. ELISpot assays were performed using 5×10^5 prestimulated antigen-RNA electroporated T-cells in 200 µl ELISpot medium per well. T-cells, which were electroporated without RNA, were used as controls. For peptide stimulation, 1 µg/ml trLT-peptides were added directly to the unelectroporated T-cells. We tested the human leukocyte antigen (HLA)-A*24:02 restricted peptide EWWRSGGFSF,[30] the HLA-A*01 restricted peptide HSQSSSSGY,[31] and the HLA-A*02 restricted peptides VIMMELNTL, SMFDEVDEAPI, and KLLEIAPNC[31] in 9 of the donors, according to their HLA-A haplotype, but did not observe specific responses against any of them, except for one HLA-A24-donor, who showed a specific response to the HLA-A24:02-restricted peptide, which was similar to the reaction upon the trLT-RNA-transfection (data not shown). After 20 h of incubation at 37°C, the ELISpot plates were washed 6 times with DPBS and stained with horseradish peroxidase (HRP)-conjugated anti-IFNγ antibody diluted 1:200 in DPBS with 0.5% FBS (PAA) for 2 h. The plates were washed again, and HRP activity was detected with a tetramethylbenzidine (TMB) substrate solution (Mabtech). ELISpot assays were evaluated using the Zeiss ELISpot Reader and the KS ELISpot 4.13.0 software. This assay was previously described and validated in detail,[26] and is well suitable to detect responses to naturally processed HLA class I-restricted responses. However, this assay is not suitable to detect HLA class II-restricted responses, since the expression of HLA class II is very low upon the T-cells, except for the few activated ones, and, more importantly, the intracellularly expressed antigens will not enter the HLA class II pathway efficiently enough.

Results

Introduction of truncated large T-antigen constructs into cmDCs

To generate a DC-based therapeutic cancer vaccine against MCC, we expressed the virus-derived oncogenic truncLT in monocyte-derived cmDCs by mRNA electroporation, to transiently introduce the antigen into the cells. For this purpose, we designed a construct, consisting of the coding sequence for the truncated form of the large T-antigen of the MCV, and a myc-tag to allow for intracellular staining [hereafter termed trLT; Figure 1(a)]. Another construct in addition contained the signal peptide of Lamp1 and the sequence of DCLamp to facilitate class II presentation in addition to class I presentation,[27] [hereafter termed trLT-DCL; Figure 1(a)]. The constructs were transcribed *in vitro* to generate mRNA for electroporation of cmDCs. At 4 h after electroporation, the expression was determined by intracellular staining of the myc-tag [Figure 1(b) and (c)]. To inhibit rapid degradation by the proteasome, the proteasome inhibitor bortezomib was added. We detected satisfying expression levels of trLT, especially when we used 15 µg of RNA/100 µl cell suspension for electroporation [Figure 1(b) and (c)]. Expression levels were only slightly increased by blocking the proteasome [Figure 1(b) and (c)]. The trLT-DCL, in contrast, was only detectable, when bortezomib was added, and only after electroporation of 15 µg of mRNA [Figure 1(b) and (c)]. This indicates that the trLT-DCL protein is rapidly degraded by the proteasome, which may lead to rapid processing and presentation of the antigenic peptides. Hence, the low protein levels we detected, did not preclude us from further using the DCLamp-modified construct. For further

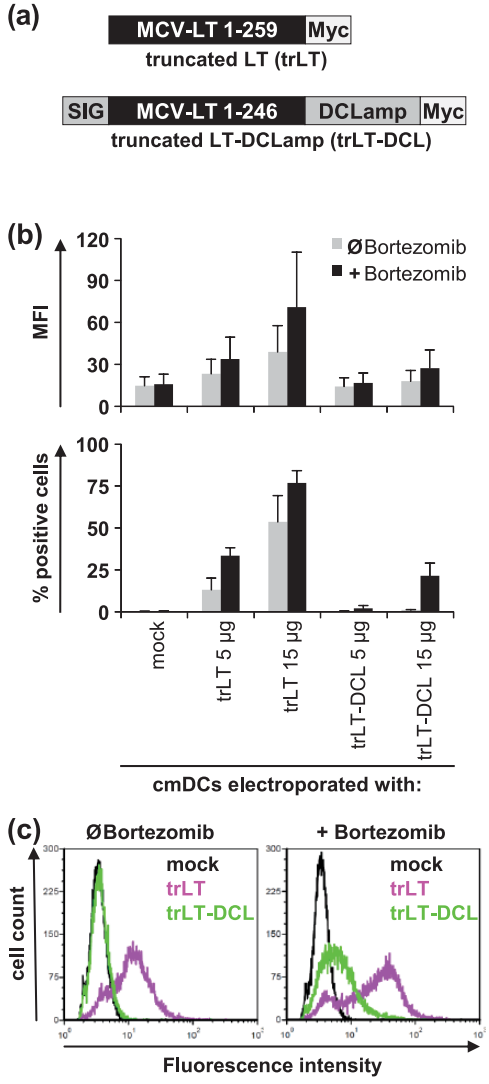

Figure 1. Introduction of trLT constructs into cmDCs.
(a) Schematic view of the antigen mRNA constructs. The truncLT (encoding amino acid 1–259) (MCV-LT 1–259) was fused C-terminally to a myc tag (Myc) for antibody detection. Additionally, the MCV-LT 1–246 was modified for MHC class II-restricted presentation by adding an N-terminal signaling peptide from Lamp1 (SIG) and C-terminally a sequence encoding human DCLamp.
(b and c) cmDCs were electroporated without RNA, with trLT RNA (trLT), or with trLT-DCLamp-RNA (trLT-DCL). The indicated quantities of RNA were used per 100 µl of cell suspension. After electroporation, the proteasome inhibitor, bortezomib, was added to achieve a better detection of the electroporated proteins. Intracellular staining was performed 4 h after electroporation with an anti-myc-tag antibody. The geometric MFI and the percentage of positive cells were measured by flow cytometry. The average of 3 independent donors ± SEM is indicated (b). One representative histogram out of 3 independent donors, showing cmDCs, electroporated with 15 µg of RNA coding for the 2 different truncLT-constructs, is depicted (c).
cmDC, cytokine-matured dendritic cell; MHC, major histocompatibility complex; MFI, mean fluorescence intensity; SEM, standard error of the mean; truncLT, truncated form of the large T-antigen.

experiments, we decided to use 15 µg of RNA coding for the trLT and the trLT-DCL constructs.

Detection of antigen-specific T-cell responses to the truncated large T-antigen in healthy donors

To overcome the limitations of conventional cmDCs concerning repetitive T-cell stimulation, we decided to use our recently developed NF-κB-activated designer DCs. Therefore, cmDCs were modified by the electroporation with constitutively active (ca)IKK-RNA to make these cells more immunogenic by strongly activating the NF-κB signaling pathway (caIKK-DCs).[25] This treatment increased the expression of several DC maturation markers and costimulatory surface molecules (CD40, CD25, CD70, OX40L, CD80, and CD86) over a period of 3 days, in comparison with cmDCs electroporated without caIKK-RNA [Figure 2(a)]. The caIKK-transfected DCs also produced higher quantities of the cytokines IL-8, TNF, IL-6, and IL-12p70, and a comparably small amount of IL-10 [Figure 2(b)]. This indicated that electroporation with caIKK-encoding mRNA further activated the cmDCs, although they had already been matured with the cytokine cocktail.

caIKK-transfected DCs were either used as such (no antigen) or constructs coding for trLT or trLT-DCL were co-electroporated (Figure 3). These DCs were then used to stimulate autologous T-cells. We used either pure $CD8^+$ T-cells or, since the DCLamp-modification should lead to presentation of helper epitopes, a 1:1 mixture of $CD4^+$ and $CD8^+$ T-cells. After 2, and after 3 rounds of stimulation, each for 1 week, these T-cells were analyzed for their reactivity against trLT. To determine the number of antigen-specific $CD8^+$ T-cells, we used a new readout-method, established in our laboratory,[26] which involved expression of the antigen within the T-cell population, resulting in major histocompatibility complex (MHC) class I presentation of the antigen. By this, the T-cells themselves acted as antigen-presenting cells and there was no need for the addition of extra target cells. For this purpose, the T-cells out of the second and the third round of stimulation were taken and electroporated with mRNAs, coding for trLT, trLT-DCL, a control-antigen with DCLamp-modification (control-DCL), or were electroporated without RNA

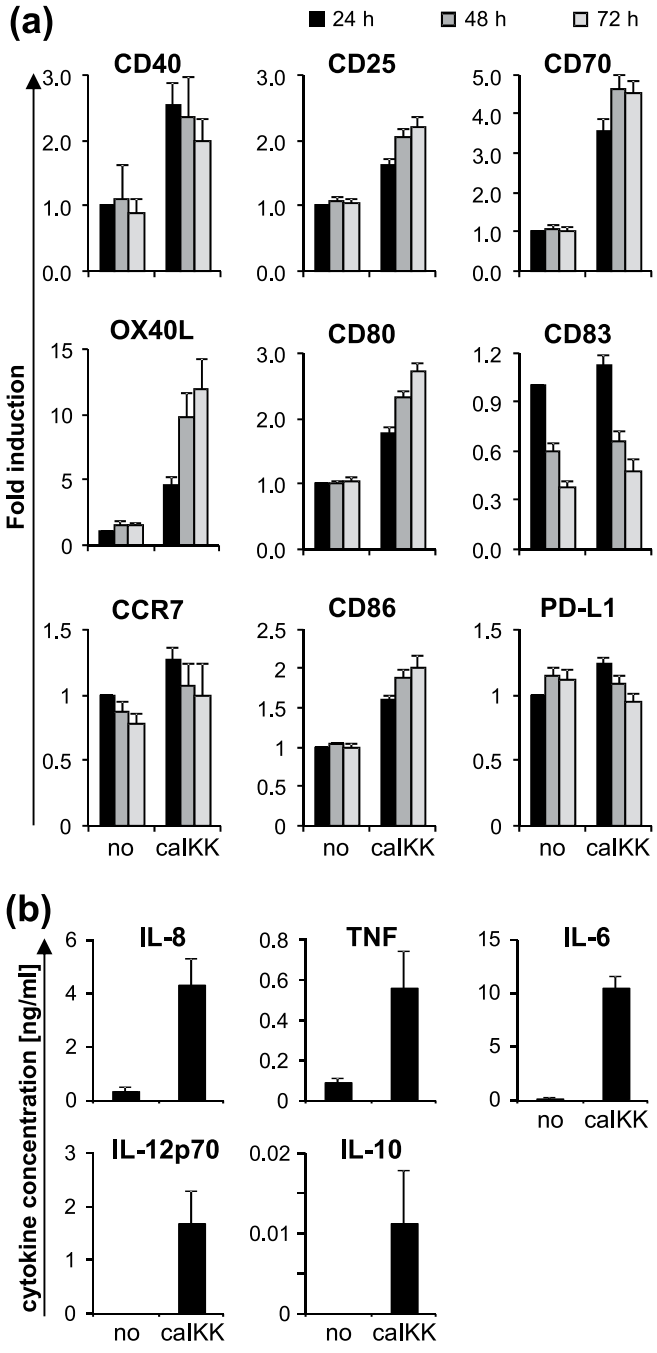

Figure 2. Electroporation of cmDCs with caIKK-RNA upregulates surface markers and induces cytokine production.

cmDCs were electroporated without (no) or with 30 µg RNA/100 µl encoding caIKK (caIKK). (a) The expression kinetics of the indicated surface markers were determined 24 h (black bars), 48 h (dark grey bars), and 72 h (light grey bars) after electroporation by flow cytometry. The bars indicate the fold induction calculated towards the 24 h control condition of 9 independent donors. The error bars indicate the SEM.

(b) The concentrations of IL-8, TNF, IL-6, IL-12p70, and IL-10 in the supernatants of the cells were determined 24 h after electroporation by a CBA. The bars indicate the mean values of 10 independent experiments. The error bars indicate the SEM.

CBA, Cytometric Bead Array; cmDC, cytokine-matured dendritic cell; IL, interleukin; SEM, standard error of the mean; TNF, tumor necrosis factor.

(mock). Production of IFNγ over-night was measured by ELISpot, indicating an antigen-specific interaction of the cells.

We observed that stimulation of $CD8^+$ T-cells [Figure 3(a)] and of the mixture of $CD4^+$ and $CD8^+$ T-cells [Figure 3(b)] with trLT-expressing

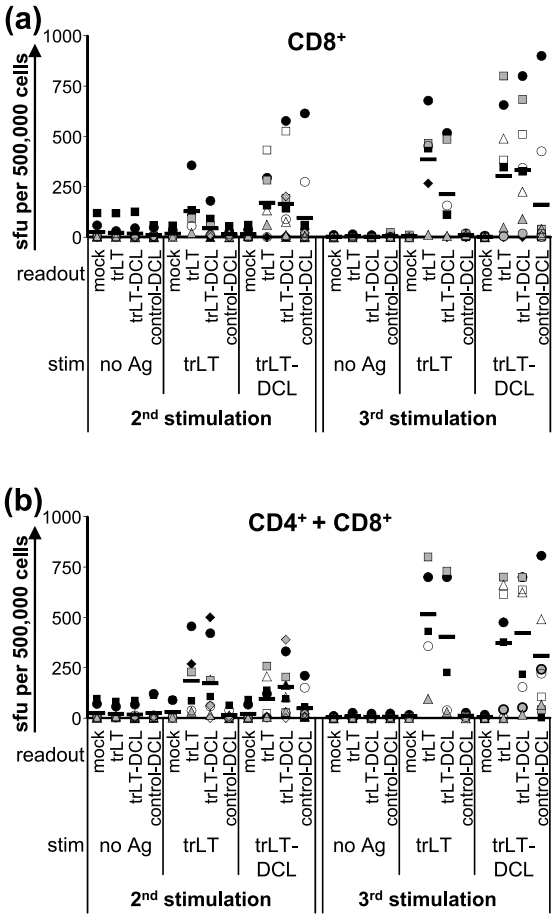

Figure 3. Detection of antigen-specific T-cell responses to the truncLT in healthy donors.
cmDCs from healthy donors were electroporated with 30 µg RNA/100 µl cell suspension coding for caIKK alone (no antigen), or in combination with the indicated amounts of truncLT-RNA, either containing a DCLamp sequence (trLT-DCL) or not (trLT). At 4 h after electroporation these DCs were used to stimulate (stim) autologous $CD8^+$ T-cells (a) or a 1:1 mixture out of $CD4^+$ and $CD8^+$ T-cells (b) for 1 week. These T-cells were restimulated twice with the same electroporated DCs, which had been cryoconserved. After the second and third round of stimulation the T-cells were examined for their reactivity towards the trLT. To provide the antigen in the readout, the T-cells were electroporated with RNA encoding the truncLT, without (trLT) or with DCLamp (trLT-DCL). As controls, the T-cells were mock-electroporated (mock) or electroporated with a control-antigen construct, also containing the DCLamp-encoding sequence (control-DCL). The electroporated T-cells were incubated overnight in an IFNγ ELISpot assay and the number of spot-forming units (sfu) per 500,000 cells, used in the assay, was determined. The data from 6–9 independent experiments are indicated; the different symbols represent different healthy donors.
cmDC, cytokine-matured dendritic cell; DC, dendritic cell; IFN, interferon; truncLT, truncated form of the large T-antigen.

IKK-DCs resulted in T-cells that produced the immunostimulatory cytokine IFNγ exclusively in response to the trLT. Especially after the third stimulation, there was a slight advantage apparent, when $CD4^+$ T-cells were also present, although the readout method was not suitable to detect the specific $CD4^+$ T-cells.

A similar picture could be seen after stimulation with trLT-DCL-electroporated caIKK-DCs, but here a response against the DCLamp-part of the construct was also observed, because the control-RNA used in the readout, that possessed the DCLamp-sequence fused to a different tumor-antigen also induced IFNγ production (Figure 3). These responses were only detected after the stimulation with trLT-DCL and are therefore exclusive responses to the DCLamp sequence. Nevertheless, they did not reach the level of the antigen-specific responses and responses directed against the trLT were also induced.

After the third round of stimulation, the responses further increased (Figure 3), and the upper detection limit of the IFNγ-ELISpot was reached for some donors. In addition, the unspecific background, observed after the second stimulation in some donors, disappeared almost completely, indicating an even higher antigen-specificity of the stimulated T-cells (Figure 3). Nevertheless, a high donor to donor variability was observed. For 2 out of 13 healthy donors we were not able to produce any antigen-specific response, whereas other donors showed very high antigen-specific T-cell responses.

These data demonstrate the high immunogenic potential of our trLT-DCs and our trLT-DCL-transfected DCs to generate antigen-specific $CD8^+$ T-cell responses in healthy donors. We believe that the usage of constructs processing the DCLamp-sequence will not have any disadvantages but helps through activating CD4 mechanisms, especially *in vivo*.

Comparison of caIKK-optimized cmDCs with conventional cmDCs

To prove that the caIKK-DCs are superior to conventional cmDCs, we compared the T-cell responses induced by 'normal' cmDCs with the ones that optimized designer caIKK-DCs could generate. We transfected cmDCs, either with the trLT-DCL alone, or together with caIKK-RNA. As above, these cells were then used to prime and stimulate autologous $CD8^+$ T-cells or a 1:1 mixture of $CD4^+$ and $CD8^+$ T-cells for week-long

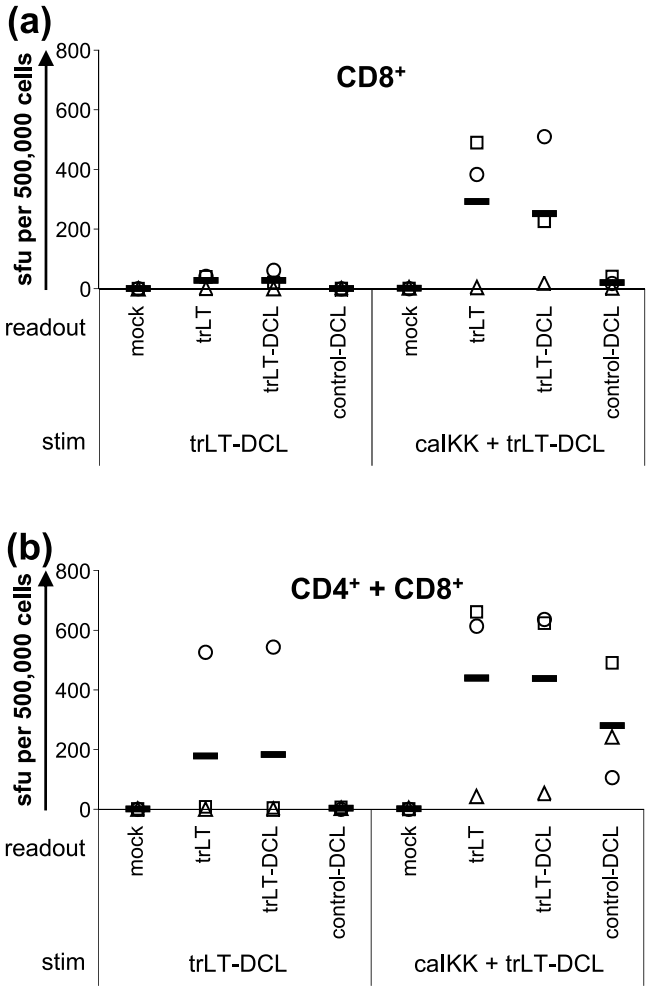

Figure 4. Comparison of caIKK-optimized cmDCs with conventional cmDCs.
cmDCs from healthy donors were electroporated with 15 µg/100 µl cell suspension truncLT-DCLamp (trLT-DCL) RNA alone or in combination with 30 µg of caIKK-RNA (caIKK). At 4 h after electroporation, these DCs were used to stimulate (stim) autologous $CD8^+$ T-cells (a) or a 1:1 mixture out of $CD4^+$ and $CD8^+$ T-cells (b) for 1 week. These T-cells were restimulated twice with the same electroporated DCs, which had been cryoconserved. After the third round of stimulation the T-cells were examined for their reactivity towards the trLT. To provide the antigen in the readout, the T-cells were electroporated with RNA encoding the truncLT, without (trLT) or with DCLamp (trLT-DCL). As controls, the T-cells were mock-electroporated (mock) or electroporated with a control-antigen construct, also containing the DCLamp-encoding sequence (control-DCL). The electroporated T-cells were incubated overnight in an IFNγ ELISpot assay. The numbers of spots per 500,000 cells used in the assay are indicated. The data from 3 independent experiments are indicated; the different symbols represent the different experiments.
cmDC, cytokine-matured dendritic cell; DC, dendritic cell; IFN, interferon; truncLT, truncated form of the large T-antigen.

rounds of stimulation. After the third round of stimulation, the T-cells were subjected to the same readout as before by transfecting them without RNA (mock), or with RNA encoding trTL, trLT-DCL, or a DCLamp-modified control antigen (control-DCL). Afterwards, secretion of IFNγ was determined (Figure 4).

When we used the 'normal' cmDCs as stimulators, we observed that the numbers of T-cells that specifically produced IFNγ was low or even undetectable if pure $CD8^+$ T-cells were stimulated [Figure 4(a)]. In contrast, we could observe high antigen-specific responses in 2 of 3 donors, when we used our optimized caIKK-transfected designer DCs [Figure 4(b)]. A similar picture was observed with the mixture of $CD4^+$ and $CD8^+$ responses [Figure 4(b)]. Here, only one donor showed an antigen-specific response if conventional cmDCs were used. This picture changed again with the use of the caIKK-optimized designer DCs. With these DCs, a clear and high antigen-specific $CD8^+$ T-cell response could be observed after stimulation with the trLT- and the

trLT-DCL-RNA in most donors [Figure 4(b)]. As described above, again a DCLamp-specific response was induced (Figures 3 and 4).

These results indicate the efficacy of our caIKK-DCs in combination with the trLT-DCL. Although this difference was not shown to be statistically significant, in combination with the results shown in Figure 2, this argues in favor of a better immunogenicity of the caIKK-transfected cmDCs. This is of obvious importance, thinking of a clinical application, where the effectiveness of DC vaccines could thus be further enhanced.

Responses against the truncated large T-antigen in patients

With respect to clinical application, it is important to know what the situation looks like in MCC patients. Due to the fact that MCC patients are usually of advanced age, they often suffer from various other concomitant diseases and sometimes have an impaired immune system. In our case the collection of patient blood proved difficult and only small volumes could be taken. Nevertheless, we wanted to test if we were able to induce immune responses against the large T-antigen also with DCs and T-cells from patients. Moreover, we also wanted to check whether the higher potential of our caIKK-DCs that we observed with material from healthy donors was also observable with patient material. Therefore, cmDCs from MCC patient-derived blood were electroporated with caIKK-RNA and the RNA coding for the trLT-DCL, either alone or in combination. These cells were then used for the stimulation of $CD8^+$ T-cells or a 1:1 mixture of $CD4^+$ and $CD8^+$ T-cells. Due to the very limited volumes of blood that could be drawn, not all conditions could be performed for each patient. After 2 rounds of stimulation, the T-cells then were analyzed using again the same readout method as above by transfecting them without RNA (mock), or with RNA encoding trLT, trLT-DCL, or a DCLamp-modified control antigen (control-DCL). Afterwards, the number of antigen-specifically IFNγ-producing cells was determined. We had the opportunity to monitor the immune response after 3 rounds of stimulation for only one patient.

We observed a very diverse picture of immune responses throughout all patients. Overall, 2 out of 5 patients did not respond at all [exemplary shown in Figure 5(a); patient #1]. This might be

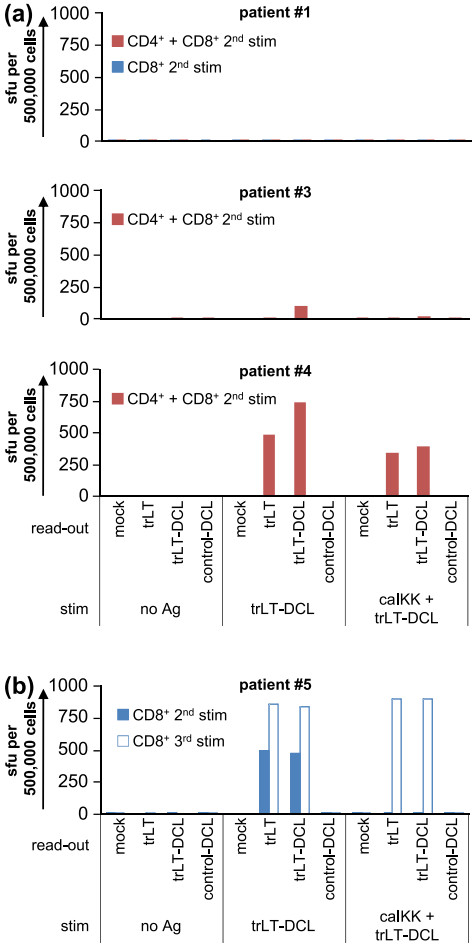

Figure 5. Responses against the trLT in patients.
cmDCs were generated out of whole blood from patients diagnosed with MCC with GM-CSF and IL-4 and matured with the standard maturation cocktail. Afterwards the cells were electroporated with caIKK-RNA or trLT-DCL-RNA or with a combination of both. At 4 h after electroporation these DCs were used to stimulate autologous $CD8^+$ T-cells (blue bars) or a 1:1 mixture out of $CD4^+$ and $CD8^+$ T-cells (red bars) for 1 week (depending on the material available). (a) These T-cells were stimulated for a second week with the same electroporated DCs which had been cryoconserved. Patient #1 is representative for patients #1 and #2 where no response was detected after 2 weekly stimulations. Patient #3 showed a weak response and patient #4 a strong response after 2 weekly stimulations. (b) For patient #5, enough cells were generated to perform a third stimulation (shown as open bars). For readout, the T-cells were electroporated with RNA encoding the truncLT, without (trLT) or with DCLamp (trLT-DCL). As controls, the T-cells were mock-electroporated (mock) or electroporated with a control-antigen construct, also containing the DCLamp-encoding sequence (control-DCL). The electroporated T-cells were incubated overnight in an IFNγ ELISpot assay. The numbers of spots per 500,000 cells used in the assay are indicated. Data from 4 different patients are shown; each panel represents one patient.
cmDC, cytokine-matured dendritic cell; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MCC, Merkel cell carcinoma; truncLT, truncated form of the large T-antigen.

due to the already exhausted phenotype of the cells and the partially very poor health status of several patients.

For patient #3, we stimulated a mixture of $CD4^+$ and $CD8^+$ T-cells and were able to detect weak responses against the trLT-DCL with 99 sfu in 500,000 T-cells prestimulated with trLT-DCL-transfected cmDCs and 18 sfu in 500,000 T-cells prestimulated with trLT-DCL and caIKK-transfected cmDCs. The negative control did not exceed 1 sfu and no response to the trLT could be observed [Figure 5(a); patient #3]. For patient #4 we could detect strong T-cell responses to the trLT, either with or without the DCLamp sequence [Figure 5(a); patient #4]. In contrast with healthy donors the additional electroporation of caIKK-RNA into the cmDCs of MCC patients did not enhance T-cell induction.

A similar pattern was observed if pure $CD8^+$ T-cells were used [Figure 5(b)]. From patient #5 we could generate enough DCs to perform a third stimulation of the T-cells. The conventional DCs induced trLT-specific T-cell responses after the second round of stimulation, no matter whether trLT or trLT-DCL was used for the readout. These responses even increased after the third round of stimulation and came close to the maximum detection limit of the ELISpot [Figure 5(b)]. Interestingly, we also observed that the caIKK-DCs caught up with the conventional DCs after the third round of stimulation [Figure 5(b)], indicating that these caIKK-transfected DCs needed more time than 'normal' designer-DCs, but then continued to expand the specific T-cells.

In summary, our results indicate that optimized designer DCs like caIKK-DCs can induce immune responses against the trLT in cells from healthy donors and MCC patients. The advantage of caIKK-transfected DCs did only emerge for the former, but not the latter. Ultimately clinical trials are required to investigate whether a DC-based vaccine represents an additional treatment option for MCC patients.

Discussion

In this study, we showed that mRNA-electroporated DCs could induce specific T-cell responses against the MCV-truncLT in blood from healthy donors and MCC patients. This is an important step towards a DC-based cellular immunotherapy against MCC. Such an intended treatment would indeed be feasible: by performing a leukapheresis procedure, over 4×10^8 DCs can regularly be generated.[32] We have performed this procedure successfully also with patients aged >75, so it would be possible with elderly MCC patients. Electroporation and subsequent cryoconservation will yield about 50% of these DCs as a vaccine,[32] resulting in enough DCs to vaccinate 6 times with 3×10^7 or 12 times with 15×10^6 living DCs. The whole process can be performed under full good manufacturing practice (GMP), as we already did several times in the context of clinical trials with melanoma patients [ClinicalTrials.gov identifiers: NCT00074230 and NCT01983748].

In therapeutic cancer vaccination, it is of great importance to choose a suitable target antigen. Therefore, the choice of a viral oncogenic antigen was quite obvious, because it is: (i) a 'foreign' antigen and thus not exposed to central self-tolerance mechanisms, (ii) it is similar in various patients, enabling its general application in MCC, and (iii) it is relevant for the oncogenic phenotype, avoiding the rise of antigen-loss variants of the tumor. The truncLT turned out to be less immunogenic than expected for a viral antigen. This may be due to the co-evolution of humans and MCV, so that the virus does not affect a healthy host while a seroprevalence of approximately 70% on average in the United States population is reported.[33] Pathogenic effects only occur if the virus integrates into the host genome and undergoes an UV-induced mutation that uncouples the cell transformation from viral replication. In addition, this protein is not the immunodominant antigen of the virus, as it is not present in the viral particles. Therefore, the truncLT has been reported to display very little immunogenicity and the generation of immune responses appears to be very difficult in healthy hosts.[31] MCV is, however, only one representative of a growing family of human polyomaviruses. Within the last few years 13 different members were found and more are probably to come (reviewed by Dalianis and Hirsch[34] and Nickeleit and Singh).[35] Most of them show a seroprevalence of >50%. Hence a cross-reactivity of large T-antigen-specific T-cells would be possible. On the other hand, the part of the large T-antigen that shows the highest homology between the different viral species is lost due to the truncation. The low number of precursors, indicated by the necessity to perform 1 or 2 restimulations *in vitro* to receive reasonable numbers of specific T-cells argues against an existing memory

response against the truncLT in most healthy donors. The high numbers of specific T-cells we observed in some patients after 2 weeks of *in vitro* stimulation could indicate, in contrast, that an existing memory response was expanded.

Because truncLT is a challenging antigen it requires highly immunogenic DCs, for example, the caIKK-optimized designer cmDCs[25] particularly in healthy volunteers without preexisting specific T-cells[31] and thus a requirement for priming. With such transfected cmDCs, T-cells, specific for this viral protein, were expanded from blood of most but not all healthy donors. The reason that some donors did not respond might be that no suitable epitopes for their individual HLA-types could be generated from the antigen. This is in line with the previous observation that T-cell responses to a comprehensive number of truncLT-epitopes could be detected exclusively in MCC patients, but not in healthy donors.[31]

The finding that this viral antigen displays no higher immunogenicity than self-antigens (e.g. Wilms' tumor protein 1[26]) is also illustrated by the observation that the DCLamp-part of the trLT-DCL construct induced similar responses for several donors. This has been described before for other highly immunogenic DCs, designed and optimized for vaccination.[36] Although it is possible that the additional activation of the NF-κB signaling pathway could already overcome the normally needed help,[37] we decided to use DCLamp-modified constructs, to possibly also induce $CD4^+$ T-cell-mediated responses.

In cellular immunotherapy, safety concerns always need to be considered. In this context, a transient expression of the truncLT by the deployed cells as well as a lack of integration into the host DNA is very important, because this protein is a tumor-driver, which could transform cells. This precludes any approach that could lead to stable expression, including DNA-based vaccination strategies, as well as viral transduction methods. On the other hand, the scarce immune responses against endogenously loaded truncLT-derived peptides[31] asks for an expression of the complete antigen, enabling the antigen processing machinery of the antigen-presenting cell (APC) to generate those epitopes that optimally fit the individual HLA haplotype. To achieve this, mRNA transfection[29] is the most suitable method.

The comparison of the caIKK-DCs with standard cmDCs revealed that the latter were also able to induce trLT-specific responses but with, in average, lower efficiency. Previously we had observed that our caIKK-DCs expanded naïve $CD8^+$ T-cells less efficiently during priming than standard cmDCs, but upon restimulation, the caIKK-DC-stimulated T-cells were expanded more efficiently and overtook the others.[25] These T-cells then reveal a highly active and probably memory-like phenotype. This would be of great advantage for the clinical application of DC vaccines against MCC.

Testing the immunogenicity of trLT-DCL-transfected DCs with MCC patient-derived material was challenging, because only small volumes of blood could be taken from the aged and often diseased patients and these patients often received treatments negatively influencing the quality of their immune cells. Nevertheless, we could induce responses which were higher after the 2nd stimulation than in healthy donor blood. However, the caIKK-DCs did not display their superiority and with the blood of one patient, we even observed a clearly lower number of specific T-cells after 2 weeks of stimulation. Here we could perform a 3rd stimulation, and then observed a much better expansion with the caIKK-DCs, resulting in a similar overall number of trLT-specific T-cells after the 3rd stimulation. Furthermore, 2 out of 5 patients showed no reaction at all, and blood we received from a patient under chemotherapy yielded no functional DCs and T-cells (data not shown). In summary, the data show that in several patients trLT-specific T-cells exist, which can be readily expanded *in vitro*. This finding is in line with the high response rate of MCC to blockade of programmed cell death protein 1 (PD-1) or its ligand PD-L1,[38] because this treatment requires preexisting cellular responses against the tumor to be efficient.

Hence, the next logical step would be to combine PD1- or PD-L1-inhibitors with therapeutic vaccination. The checkpoint inhibitors as monotherapy already showed high benefits in the overall survival, and because there are no sufficient standard therapies available, experts in this field raised the justified request to use anti-PD-L1 or anti-PD-1 antibodies as first line treatment against MCC from now on, even without further clinical phase II or III trials, because the results obtained so far are overwhelming.[19] Unfortunately, still not all patients responded to this kind of therapy.[38] For malignant melanoma, it was already shown that the combination of DC-based vaccines and checkpoint inhibitors seems to increase the response

rates.[39] Additionally, the combinatorial approach might allow reducing the dose of checkpoint-blocking antibody. Especially in the aged and often multi-morbid patients any reduction of the side effects of this treatment would be desirable.

The clear-cut and now well understood mechanism of how MCV is inducing MCC makes the truncLT a good role model for virus-derived oncogenic drivers, but it is not the only one. Also in the genesis of other human tumors, viruses play an important role, and the aspect of other viral antigens as targets for immunotherapy with DCs or other vaccine strategies is of great importance.[40] Viral antigens expressed in human tumors possess exclusive features, which make them an ideal target for immunotherapy, and probably patients suffering from other tumor types could benefit from this concept.

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Conflict of interest statement

The authors declare the following potential conflict of interest: REV, GS, NS, and JD are named as inventors on a patent on caIKK-RNA-electroporated DCs (WO/2012/055551), which is held by the University of Erlangen-Nuremberg, Erlangen, Germany.

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