

Steven De Vleeschouwer · Mohammed Arredouani
Martine Adé · Pascal Cadot · Elke Vermassen
Jan. L. Ceuppens · Stefaan W. Van Gool

Uptake and presentation of malignant glioma tumor cell lysates by monocyte-derived dendritic cells

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Abstract Malignant glioma of the CNS is a tumor with a very bad prognosis. Development of adjuvant immunotherapy is hampered by interindividual and intratumoral antigenic heterogeneity of gliomas. To evaluate feasibility of tumor vaccination with (autologous) tumor cells, we have studied uptake of tumor cell lysates by dendritic cells (DCs), and the T-cell stimulatory capacity of the loaded DCs. DCs are professional antigen-presenting cells, which have already been used as natural adjuvants to initiate immune responses in human cancer. An efficacious uptake of tumor cell proteins, followed by processing and presentation of tumor-associated antigens by the DCs, is indeed one of the prerequisites for a potent and specific stimulation of T lymphocytes. Human monocytes were differentiated *in vitro* to immature DCs, and these were loaded with FITC-labeled tumor cell proteins. Uptake of the tumor cell proteins and presentation of antigens in the context of both MHC

class I and II could be demonstrated using FACS analysis and confocal microscopy. After further maturation, the loaded DCs had the capacity to induce specific T-cell cytotoxic activity against tumor cells. We conclude that DCs loaded with crude tumor lysate are efficacious antigen-presenting cells able to initiate a T-cell response against malignant glioma tumor cells.

Keywords Confocal microscopy · Cytotoxicity · Dendritic cells · Flow cytometry · Malignant glioma · Tumor proteins

S. De Vleeschouwer · M. Arredouani
M. Adé · P. Cadot · Jan. L. Ceuppens
Laboratory of Experimental Immunology,
Catholic University of Leuven, Leuven, Belgium

E. Vermassen
Laboratory of Physiology, Catholic University of Leuven,
Leuven, Belgium

S. De Vleeschouwer
Department of Neurosurgery, Catholic University of Leuven,
Leuven, Belgium

S. W. Van Gool
Department of Pediatrics, Catholic University of Leuven,
Leuven, Belgium

M. Arredouani
Department of Environmental Health,
Harvard School of Public Health, Boston,
Massachusetts, USA

S. W. Van Gool (✉)
University Hospital Gasthuisberg, Herestraat 49,
3000 Leuven, Belgium
E-mail: Stefaan.VanGool@uz.kuleuven.ac.be
Tel.: +32-16-332211
Fax: +32-16-343842

Introduction

Malignant gliomas can be divided into grade III anaplastic glioma and grade IV glioblastoma multiforme (GBM). The yearly incidence is 2.5/100,000 [13]. In spite of modern neurosurgery, new developments in radiotherapy, and the availability of combinations of chemotherapeutic agents, some of them being newly developed, the prognosis of malignant glioma remains poor. Adjuvant immunotherapy is therefore an option to improve survival chances.

Several trials of immunotherapy with tumor vaccines were recently set up to treat patients with melanoma, prostate cancer, renal cell carcinoma, multiple myeloma, lymphoma, breast and ovarian cancer, and pediatric malignancies [28]. The technology of *in vitro* DC differentiation out of peripheral blood monocytes [46] or CD34⁺ stem cells [52] made DCs available for use as therapeutic vaccines in clinical practice. In most of these trials, well-defined tumor-associated peptides have been used to load autologous DCs *ex vivo*, which were then injected into the patient. The use of peptides for loading DCs allowed the investigators to set up specific immune monitoring during and after vaccination [23].

Although located in a so-called immune privileged environment, there is a strong interaction between the malignant glioma and the immune system. Monocytes

and T lymphocytes infiltrate the tumor [6]. However, the tumor cells produce TGF- β [30], IL-10 [26], PGE₂ [21], and other immune suppressive factors [37], thereby creating an immune suppressive local environment [16] and even systemic immune suppression [18]. Several tumor antigens have been described on glioma tumor cells, including EGFRvIII isoform [35], gp240 [31], tenascin [54], survivin [57], SART 1 [27], IL-13R α 2 chain [38], and melanoma-associated antigens like tyrosinase, tyrosinase-related protein 1 and 2, gp100, MAGE-1, and MAGE-3 [12]. One particular drawback for the development of DC vaccination against malignant gliomas, however, is the interindividual and intratumoral antigenic heterogeneity of gliomas [5, 12, 48]. Therefore, preclinical investigations for the development of therapeutic vaccines against malignant gliomas were based on the use of autologous DCs loaded with a mixture of acid-eluted peptides [33], apoptotic bodies [56], or tumor cell lysates [24]. Similarly, clinical trials have been conducted using autologous DCs loaded with acid-eluted peptides [34, 59], DCs fused with tumor cells [29], and DCs loaded with tumor lysates [14, 55, 58].

As part of the development of a DC-based vaccination program against malignant gliomas, we focused in this study on the ability of DCs to take up proteins from tumor cell lysates. We monitored the uptake and presentation of FITC-labeled protein fragments from tumor cell lysates in the context of MHC class II molecules, and, more importantly, cross-presentation in the context of MHC class I molecules. The latter is required to induce CD8⁺ cytotoxic T lymphocyte (CTL) activity. As a read-out of DC immunogenicity, we analyzed the *in vitro* induction of tumor-specific cytotoxic activity when T cells were stimulated with autologous DCs loaded with tumor cell lysate.

Material and methods

Generation of monocyte-derived dendritic cells

The study was approved by the local ethical committee, and informed consent was obtained from all individuals who gave material. Peripheral blood mononuclear cells (PBMCs) were isolated from blood from healthy volunteers and glioblastoma patients using Lymphoprep (Nycomed Pharma, Oslo, Norway). Subsequently, the monocyte fraction was enriched using plastic adherence as described previously [45]. The monocytes were cultured in six-well plates (Greiner Bio-One; Sarah SE, Longwood, FL, USA) at a concentration of 10⁷ cells per milliliter in short-term culture medium. This medium consisted of RPMI 1640 (BioWhittaker, Verviers, Belgium), supplemented with glutamine (2 mM), penicillin (200 U/ml), streptomycin (200 μ g/ml) (all from Cambrex, East Rutherford, NJ, USA), and 1% autologous plasma. The monocytes were differentiated into immature DCs by culture in the presence of recombinant human IL-4 (20 ng/ml; PeproTech, Rocky Hill, NY,

USA) and rGM-CSF (1,000 U/ml; Novartis Pharma, Brussels, Belgium) for 7 days. Half of the culture medium was refreshed at day 3.

After 7 days, the immature DCs were collected. Maturation was induced with a cytokine mixture consisting of rTNF- α (120 ng/ml; Strathmann Biotec, Hamburg, Germany), rIL-1 β (120 ng/ml; Strathmann Biotec), and PGE₂ (20 μ g/ml; Pharmacia, Puurs, Belgium) [50].

Glioblastoma (GBM) cell cultures

The GBM cell lines A172 and U251 (kindly provided by J.E.A. Wolff, Regensburg, Germany) were cultured in tissue culture flasks (Nunc, Roskilde, Denmark). The long-term cell culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), nonessential amino acids (diluted 1:100) (Cambrex), glutamine (2 mM), penicillin (200 U/ml), and streptomycin (200 μ g/ml), β -mercaptoethanol (7 \times 10⁻⁴%), and amphotericin B (1.25 μ g/ml; Sigma-Aldrich, St Louis, MO, USA). Primary GBM tumor cells were obtained out of freshly resected tumor samples by culturing small pieces of tissue in medium. Tumor cells from early passage cultures (<10 passages) were characterized by gliofibrillary acid protein and vimentin monolayer staining.

Tumor lysate

Freshly resected glioblastoma specimens were snap-frozen and stored in liquid nitrogen. After thawing, each tumor specimen was minced into very small pieces (less than 0.5 mm³) in physiological saline. The pieces were further broken down mechanically by continuous blunt graining until a homogeneous suspension was reached. This suspension was further homogenized by repetitive pipetting. Thereafter, six snap-freeze/thaw cycles were performed by rapid freezing in liquid nitrogen and immediate thawing at 37°C. Finally, the lysate was filtered using a 70- μ m cell strainer (BD Falcon; BD Biosciences Europe, Erembodegem, Belgium). The protein content was measured using the Coomassie Blue assay [7]. Cell death was verified by trypan blue exclusion. When GBM tumor cell lines were used to prepare the lysate, tumor cells were harvested, six snap-freeze/thaw cycles were performed, and lysate was filtered through the 70- μ m cell strainer. The lysate was kept in liquid nitrogen until use.

In some experiments, tumor cell proteins in the lysate were labeled with FITC. For this, 1–2 mg of lysate protein was transferred into labeling buffer (0.05 M boric acid, 0.15 M NaCl, pH 9.2) by salting-out chromatography (Econo 10 DG column; Biorad, Richmond, CA, USA). Thereafter, 20 μ l of FITC solution (5 mg/ml in DMSO [B/Braun, Boulogne, France]) was added per milligram of tumor protein and incubated for 2 h at

room temperature with gentle and continuous stirring. Unbound FITC was removed by gel filtration in an Econo 10 DG column previously equilibrated with phosphate buffer solution (DPBS; BioWhittaker). The obtained preparation was kept at 4°C until use.

FACS analysis

PE-labeled anti-MHC class I and anti-MHC class II mAbs were purchased from BD Biosciences Pharmingen (San Jose, CA, USA) and used according to the manufacturer's instructions. DCs that were loaded with FITC-labeled tumor lysate were washed twice with RPMI 1640 and stained with PE-labeled anti-MHC class II mAbs in the presence of neutralizing plasma (10% diluted in PBS). The cells were washed again with cold PBS and fixed in 1% paraformaldehyde before analysis. FACS analysis was performed using CellQuest software (BD, Mountain View, CA, USA).

Confocal microscopy

Immature DCs were transferred in a two-chambered borosilicate coverglass system (Lab-Tek; Nalge Nunc International, Naperville, IL, USA), at a cell density of 0.2×10^6 per chamber, and were loaded in situ with 60 µg FITC-labeled tumor cell proteins per million DCs. After 30 min the medium was refreshed. DCs were analyzed using a Zeiss confocal laser scanning microscope LSM510 (Carl Zeiss, Jena, Germany) for another 90 min to localize the labeled protein fragments within the DCs. To visualize the FITC-labeled protein fragments, FITC was excited at 488 nm using an argon laser, and the emitted fluorescent light was collected by a photomultiplier after passage through a 505-nm LP filter.

In other experiments, 10^6 immature DCs in 1 ml short-term medium were loaded with 60 µg FITC-labeled tumor homogenate for 30 min at 37°C in 5% CO₂. Afterward, loaded DCs were washed, and 0.5×10^6 cells were cultured in 1 ml per well of a 24-well plate for 24 h in the presence of rTNF- α , rIL-1 β , and PGE₂. After the incubation, cells were harvested and stained with PE-labeled anti-MHC class I or anti-MHC class II mAb in the presence of neutralizing plasma. As control, the cells were also stained with PE-labeled IgG1, anti-CD80, or anti-CD83 (all from BD Biosciences Pharmingen). Cells were fixed with paraformaldehyde and plated in two-chambered borosilicate coverglass systems at a concentration of 0.3×10^6 DC per chamber. For double labeling of the FITC-labeled protein fraction (green) and the PE-labeled anti-MHC mAb on the DCs (red), the emission light of FITC and PE was alternately recorded by a Zeiss confocal laser scanning microscope LSM510 equipped with a two-channel recording configuration. Based on the excitation and emission wavelength characteristics of the two fluorophores (see <http://fluorescence.nexus-solutions.net>),

FITC was excited by an argon laser at 488 nm, and the emitted light was collected by a photomultiplier after passage through a 475–525-nm BP filter. The fluorescent light of PE was collected after passage through a 505-nm LP filter following excitation at 543 nm using a helium-neon laser. The LSM 510 software of the confocal microscope enabled us to screen automatically for color overlay (yellow).

T cell isolation

Highly purified T cells from donors or patients were isolated as described previously [8]. Briefly, PBMCs were isolated by centrifugation of blood on Lymphoprep, and resuspended in short-term culture medium and inactivated autologous plasma at 10%. T cells were further purified using a complement-mediated depletion of all non-T cells with lympho-KWIK-T (One Lambda, Los Angeles, CA, USA). The percentage of contaminating NK cells at baseline and after two stimulations was less than 3%.

In vitro priming of autologous T cells

Immature DCs were loaded overnight with lysates from autologous tumors (100 µg tumor proteins per 10^6 DCs) or lysates from A172 or U251 cells (DC to tumor ratio of 1:1). Addition of the maturation cytokine mixture at the moment of loading started maturation of DCs. T cells were stimulated with autologous mature loaded DCs at a ratio of 10:1. During the first stimulation of 7 days, rIL-6 (10 ng/ml; Peprotech, Rocky Hill, NJ, USA) and rIL-12 (10 ng/ml; Genetics Institute, Cambridge, MA, USA) were added. Cells were harvested and 2×10^6 T cells were cultured again for 7 days with 0.2×10^6 newly differentiated and loaded DCs in the presence of rIL-2 (10 U/ml; Roche Diagnostics, Mannheim, Germany) and rIL-7 (5 ng/ml; Peprotech) [11, 25, 53]. At the end of the second stimulation, T cells were harvested and their effector function was analyzed.

Flow cytometric cytotoxicity (FCC) assay

Target cells (GBM cells or autologous T-cell blasts) were stained with the fluorescent probe CTO (Cell-Tracker Orange CMTMR; Molecular Probes Europe, Leiden, The Netherlands). T-cell blasts were generated from autologous T cells by stimulation with rIL-2 (10 U/ml) and phytohemagglutinin (1 µg/ml, PHA; Rurex Isotech, Dartford, England). For staining of target cells in the culture flasks, CTO was used in RPMI 1640 at a final concentration of 5 µM, according to the manufacturer's prescriptions. A total of 20×10^5 target cells were added to increasing amounts of CTL in short-term medium, and cultured for 3 h at 37°C in 5% CO₂. Afterwards, cells in each vial were washed

twice in modified HEPES buffer (10 mM HEPES [1 M, BioWhittaker], 140 mM NaCl [Merck, Darmstadt, Germany], and 2.5 mM CaCl₂ [Janssen Chimica, Geel, Belgium]), and stained with annexin V-FITC (BD Biosciences Pharmingen Europe) and 7-amino-actinomycin D (7-AAD; Calbiochem, La Jolla, CA, USA) according to the manufacturer's instructions. Target cells alone without staining were used as control for autofluorescence. Based on their uptake of CTO, target cells, but not effector cells, were detected as positive cells in the FL-2 channel (emission of 566 nm), and were gated. CTO-labeled target cells that were cultured without effectors were used as baseline for the assessment of T-cell-mediated cytotoxicity. Induction of target cell death by effector T cells was measured by analyzing the CTO+ target cells that became positive for annexin V (emission of 530 nm measured in FL-1 channel) and 7-AAD (emission of 655 nm measured in FL-3 channel) (AnnV+/7AAD+). Specific cytotoxicity was calculated for each E/T ratio of each condition as follows: % specific cytotoxicity = (% AnnV+/7AAD+ target cells)_{exp} - (% AnnV+/7AAD+ target cells)_{baseline}. In some cytotoxicity assays, a blocking anti-MHC class I mAb HB95 (ATCC, Manassas, VA, USA) was used. For this, target cells were preincubated for 45 min with HB95 used at 10 µg/ml.

Results

Uptake of FITC-labeled proteins from tumor cell lysates by immature DCs

In a first series of experiments, tumor cell proteins labeled with FITC were used to load immature monocyte-derived DCs. For this, 0.5×10^6 immature DCs were incubated at 37°C with 60 µg tumor cell proteins in 500 µl RPMI 1640 for 5, 15, 30, and 90 min. The control condition consisted of unloaded DCs incubated at 37°C for 90 min. To demonstrate that metabolic activity is required for the uptake of the tumor cell proteins, a separate vial with DCs and FITC-labeled tumor cell proteins was kept at 4°C for 90 min. After the incubation

period, DCs were stained with PE-labeled anti-MHC class II mAb. The green fluorescent intensity of MHC class II⁺ DCs, which reflects binding and uptake of FITC-labeled proteins, was followed over time (Fig. 1). We found a continuous increase of the mean fluorescence intensity (MFI) in the FL-1 (FITC green fluorescence) channel when DCs were incubated with FITC-labeled tumor cell proteins. Incubation overnight further increased the MFI (data not shown). This shift was only observed when cells were kept in the incubator at 37°C. Incubation of the DCs at 4°C for 90 min resulted in a limited increase of MFI, most likely corresponding to sticking of the FITC-labeled proteins to the DC cell surface, without intracellular uptake.

Analysis of DC loading with FITC-labeled tumor cell proteins, by confocal microscopy

We next analyzed the uptake and cellular distribution of the FITC-labeled proteins in the DC cultures. For this, immature DCs were incubated with FITC-labeled tumor cell proteins for 30 min. Afterward, the medium was refreshed. The uptake and cellular distribution of the FITC-labeled proteins was analyzed during the following 90 min with the confocal microscope (Fig. 2). In the interval between 30 and 70 min after the start of the incubation, at first a diffuse binding of tumor cell proteins to the cell surface followed by progressive intracellular uptake was seen. There was a clear presence of FITC-labeled protein fractions in the cytosol. In the interval between 60 and 90 min after the start of the incubation, we noticed a progressive and polarized reappearance of labeled tumor cell proteins on the DC cell surface. Thereafter, no further changes occurred.

FITC-labeled tumor cell protein fragments colocalize with MHC class I and MHC class II molecules on DCs

For adequate stimulation of CD8⁺ and CD4⁺ T cells, antigenic peptides have to be presented in the context of autologous MHC class I and MHC class II molecules,

Fig. 1 FACS analysis of uptake of labeled proteins from tumor cell lysate by immature DCs. FITC-labeled proteins from tumor cell lysate were added to suspensions of immature DCs (60 µg per 10⁶ DCs). These were either cultured at 37°C (left) or kept at 4°C (right). At different time points, cells were harvested and stained with PE-labeled anti-MHC class II mAb. The shift of the green fluorescence intensity on the MHC class II⁺ cells was analyzed by FACS. The data are representative of three independent experiments

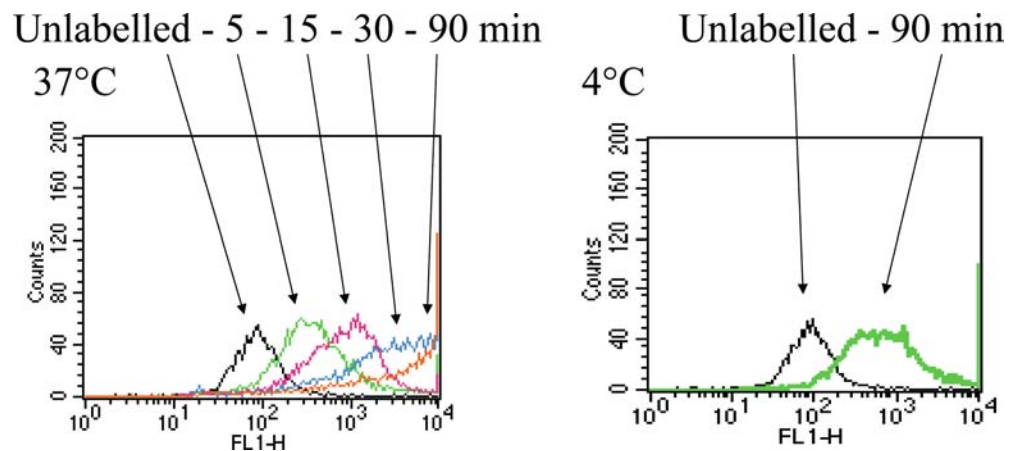
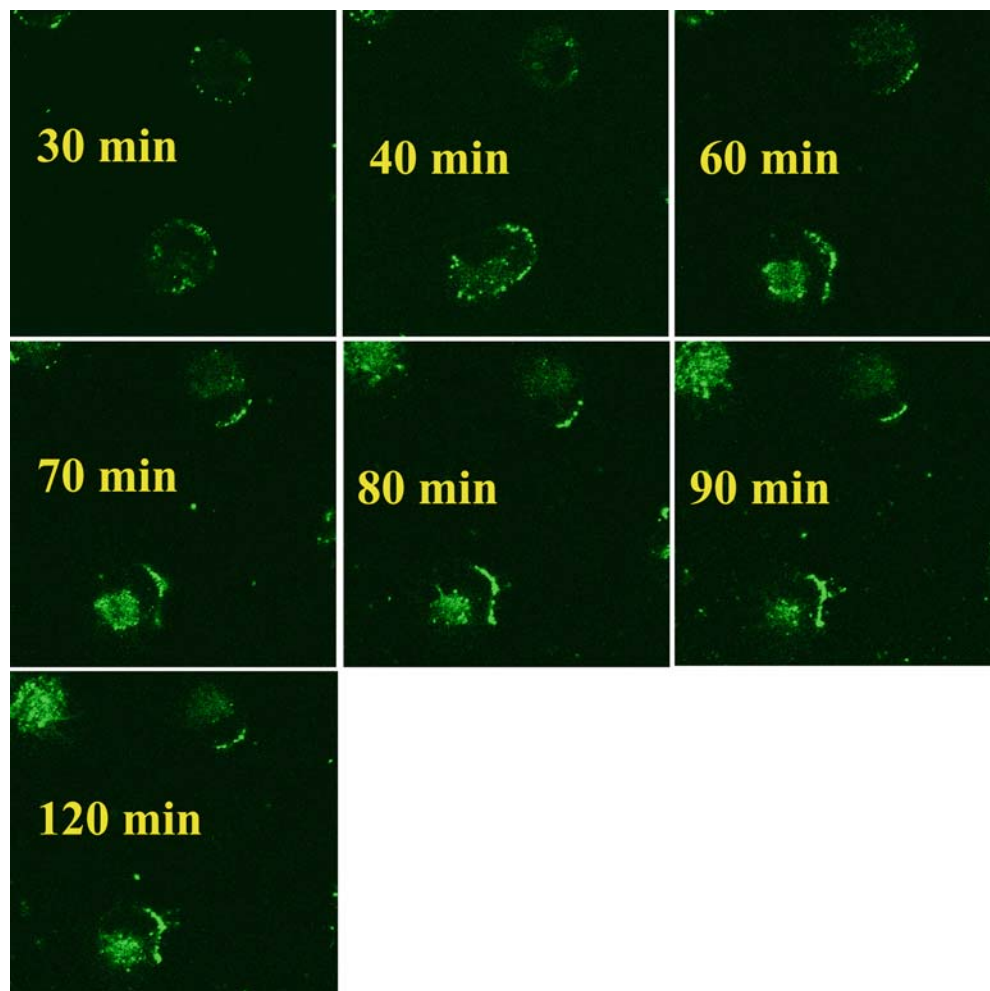


Fig. 2 Confocal microscopy analysis of the uptake of FITC-labeled proteins from tumor cells by DCs. Immature DCs were cultured during 30 min in the presence of FITC-labeled proteins from tumor cells, as explained in Fig. 1. Afterward, the medium was refreshed, and the uptake of the proteins was visualized at different time points with confocal microscopy. The time points refer to the start of the loading. The pictures are representative of two independent experiments



respectively. To demonstrate at least colocalization between the FITC-stained tumor cell proteins and MHC molecules, DCs loaded for 30 min with FITC-labeled tumor cell lysates as described in the previous paragraph, were stained with PE-labeled anti-MHC mAb. To assess colocalization, confocal microscopy was used and cells were screened for color overlay (FITC and PE) visible as yellow (Fig. 3a). After 30 min, colocalization of FITC-labeled tumor protein fragments and PE-stained MHC class II molecules was obvious. After 90 min, yellow patches on the cell surface were also noticed on the DC stained for MHC class I molecules, thus demonstrating colocalization of tumor protein fragments and MHC class I molecules.

In a second series of experiments, DCs were loaded for 30 min with FITC-labeled tumor cell lysate. Afterward, DCs were washed and further cultured in the presence of the maturation cytokine cocktail for 24 h, which in control experiments induced maturation of DCs as evidenced by CD83 expression and up-regulation of HLA-DR, CD86, and CD80 (data not shown). We again were able to detect clear yellow patches of colocalization of the FITC-labeled tumor cell protein fragments and PE-labeled mAb to both MHC class I

and MHC class II (Fig. 3b). In these experiments, PE-labeled control IgG1 mAb did not result in positive staining or overlay (not shown). When the loaded DCs were stained with PE-labeled anti-CD80 or anti-CD83, staining but without overlay could be detected (not shown).

Immunogenicity of DCs loaded with GBM tumor cell lysates

The most relevant assessment of immunogenicity of DCs loaded with tumor cell proteins is their capacity to stimulate autologous T cells and to induce T-cell cytotoxicity against the tumor cells. We therefore performed *in vitro* experiments, in which T cells were stimulated with loaded autologous DCs for 7 days in the presence of rIL-6 and rIL-12, and for another 7 days in the presence of rIL-2 and rIL-7 [11, 25, 53]. At the end of these stimulations, the T-cell activation marker CD25 was expressed by both CD8⁺ and the reciprocal CD8⁻ (being the CD4⁺) T cells when T cells were stimulated with loaded DCs (Fig. 4). T cells cocultured with unloaded DCs in the presence of cytokines showed

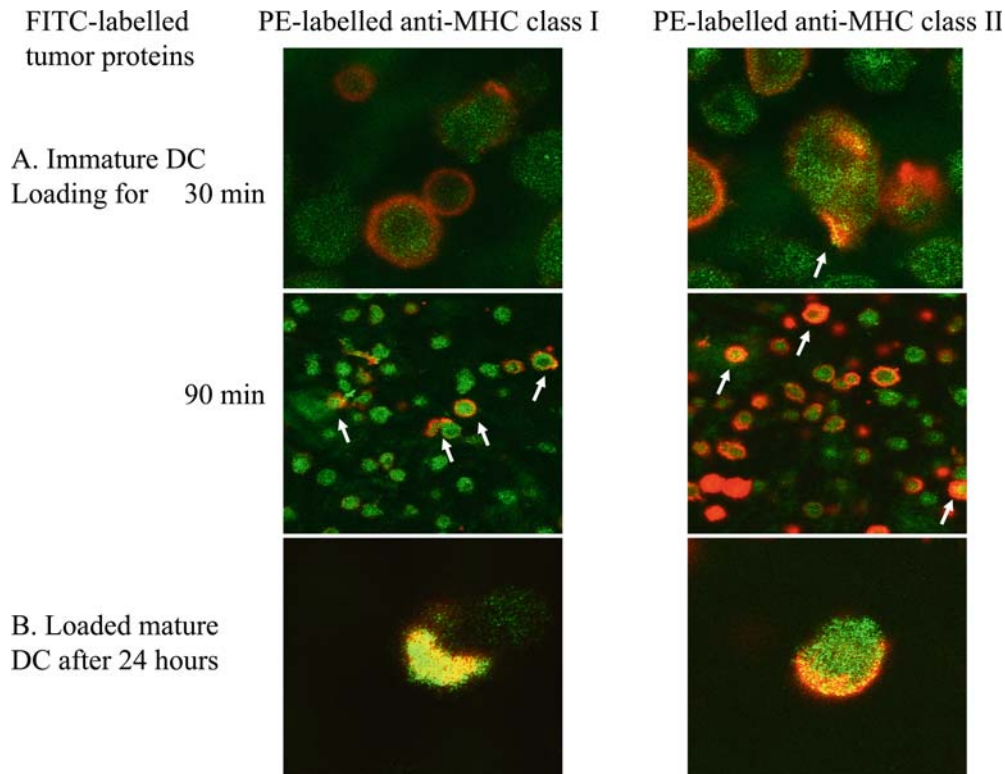


Fig. 3 Colocalization of FITC-labeled proteins from lysed tumor cells and MHC class I and class II molecules. **a** DCs were loaded during 30 or 90 min with FITC-labeled proteins from lysed tumor cells, as explained in Fig. 1. Afterward, the cells were washed and stained with PE-labeled anti-MHC mAb. Color overlay of FITC with PE results in a yellow color, and this was assessed with confocal microscopy at two different time points. Results are representative of two independent experiments. **b** Immature DCs were loaded during 30 min with FITC-labeled proteins from lysed tumor cells. Cells were washed and DC maturation was induced by culture with rIL-1, rTNF- α , and PGE₂ for 24 h. Colocalization of the FITC-labeled proteins (green) and PE-labeled anti-MHC class I and class II mAbs (red) was assessed with confocal microscopy. Colocalization of green and red fluorescence results in a yellow color. Results shown are representative of five independent experiments

up-regulation of CD25 in the CD8⁻ cell population, although less than in the experimental condition, but not in the CD8⁺ cell population. T cells from a volunteer were stimulated in the presence of autologous DCs loaded with lysate from A172 GBM cells. The volunteer matched with the A172 cell line for the MHC class I molecules HLA-A3 and HLA-B7. As shown in Fig. 5a, T cells stimulated in this way were then able to kill A172 target cells. T cells that had been cultured with unloaded DCs and cytokines, or in the presence of cytokines alone, had a lower cytotoxic activity against A172 cells. In a similar experiment, we stimulated T cells from a patient with autologous DCs loaded with lysate from U251 GBM cells. There was compatibility at the level of HLA-A2. Also in this experiment, T cells stimulated with loaded DCs had cytotoxic activity against U251 target cells in contrast to T cells that were cultured in the presence of unloaded DCs and cytokines (Fig. 5b). We

finally studied generation of CTL activity against autologous tumor cells. DCs, T cells, and tumor cells from a patient with malignant glioma were isolated. T cells that were stimulated with loaded autologous DCs had higher cytotoxic activity against autologous GBM tumor cells than T cells that were cocultured with unloaded autologous DCs in the presence of cytokines (Fig. 5c). To demonstrate that the killing was dependent on the interaction between the cytotoxic T cells and MHC class I molecules on the target cells, we added blocking anti-MHC class I mAb HB95 to the target cell prior to the effector phase. This mAb was not cytotoxic to the tumor cells, and could effectively block MHC class I staining of the tumor cells (data not shown). Blocking MHC class I molecules on the target cells largely prevented cell killing induced by effector T cells (Fig. 5d).

To demonstrate the specificity of tumor cell killing by T cells stimulated with lysate-pulsed DCs, we studied effector responses against matched (at an HLA A allele) or mismatched GBM tumor cells (Fig. 6a). T cells stimulated with autologous DCs loaded with lysates from GBM tumor cell lines with matched HLA A allele, could generate cytotoxic activity against these HLA A-matched tumor cells. When DCs were loaded with lysates from MHC class I-mismatched GBM tumor cells, we could not detect generation of cytotoxic activity against these target cells. To further demonstrate tumor cell-specific killing, we compared the cytotoxic activity of T cells against GBM tumor cells and autologous T-cell blasts in a complete autologous setting (T cells, DCs, and both target cells derived from patients). As shown in

Fig. 4 Stimulated CD8⁻ and CD8⁺ T cells express CD25 activation marker. T cells were cultured with autologous DCs loaded with tumor cell proteins, in the presence of rIL-6/rIL-12 and afterward in the presence of rIL-2/rIL-7, as described in "Material and methods." T cells were cultured with unloaded DC and cytokines as control condition. Before and at the end of the second stimulation, T cells were stained for CD8 (FITC) and CD25 (PE) expression

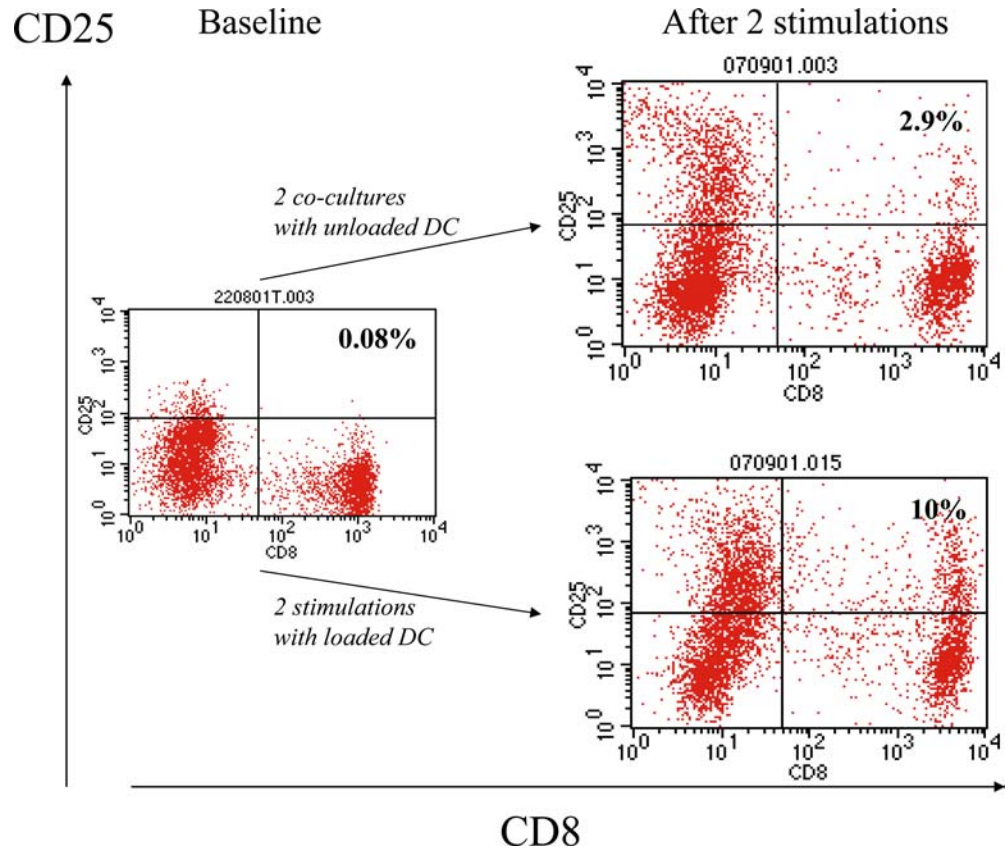


Fig. 6b, the cytotoxic activity of effector cells (i.e., T cells stimulated for 7 days with loaded autologous DCs in the presence of rIL-6/rIL-12 and restimulated for 7 days with loaded autologous DCs in the presence of rIL-2/rIL-7) against T-cell blasts was less than half of the cytotoxic activity against the GBM tumor cells.

Discussion

This is the first report using confocal microscopy for the investigation of the colocalization of FITC-conjugated GBM tumor cell proteins with MHC molecules on the cell surface of DCs. The use of GBM tumor cell proteins, directly labeled with FITC and used in uptake assays, enabled us to analyze kinetically their uptake, intracellular distribution, and reexposure on the membrane of DCs. FACS analysis revealed (after an initial phase of membrane binding which also occurred at 4°C) the progressive intracellular uptake of tumor cell proteins, requiring DC metabolic activity. Whether the uptake is based on phagocytosis of cell fragments [2], macropinocytosis of soluble antigen [44], or uptake via specific receptors like DC-SIGN [19] or FcγR [22] is not clear yet. The polarized reappearance of FITC-labeled protein fragments on the cell surface is highly suggestive for presentation in a MHC context [4]. Unlike other antigen-presenting cells, DCs are actively involved in formation of the immunological synapse through rear-

angement of their actin cytoskeleton, thereby allowing them to activate resting T cells [1].

Specific priming of naïve T cells by DCs implies the presentation of the tumor antigens in the context of MHC molecules. Therefore, we analyzed colocalization of FITC-labeled protein fragments and anti-MHC class I and class II mAb, both conjugated with PE. The appearance of a yellow overlay color after 30 and 90 min for MHC class II and MHC class I staining, respectively, supports the hypothesis of MHC-related presentation. The kinetic and the intensity of the yellow overlay color suggest that antigen uptake and cross-presentation of exogenous proteins by MHC class I is less efficient than loading of MHC class II molecules. However, the confocal microscopy assay can not quantify the extent of loading in the different MHC molecules. Presentation of exogenous peptides in a MHC class II context by DCs is a well-documented phenomenon. The efficiency of tumor antigen presentation in the context of MHC class I molecules could be functionally demonstrated after stimulation, by the up-regulation of the activation marker CD25 on the CD8⁺ T cells and by the generation of cytotoxic activity against MHC class I-compatible target cells. Presentation of peptides in a MHC class I context is believed to take place via another pathway, separate from the pathway of presentation in a MHC class II context. Several reports, however, have shown that these pathways are not completely distinct. DCs are able to cross-present exogenous peptides in a MHC class

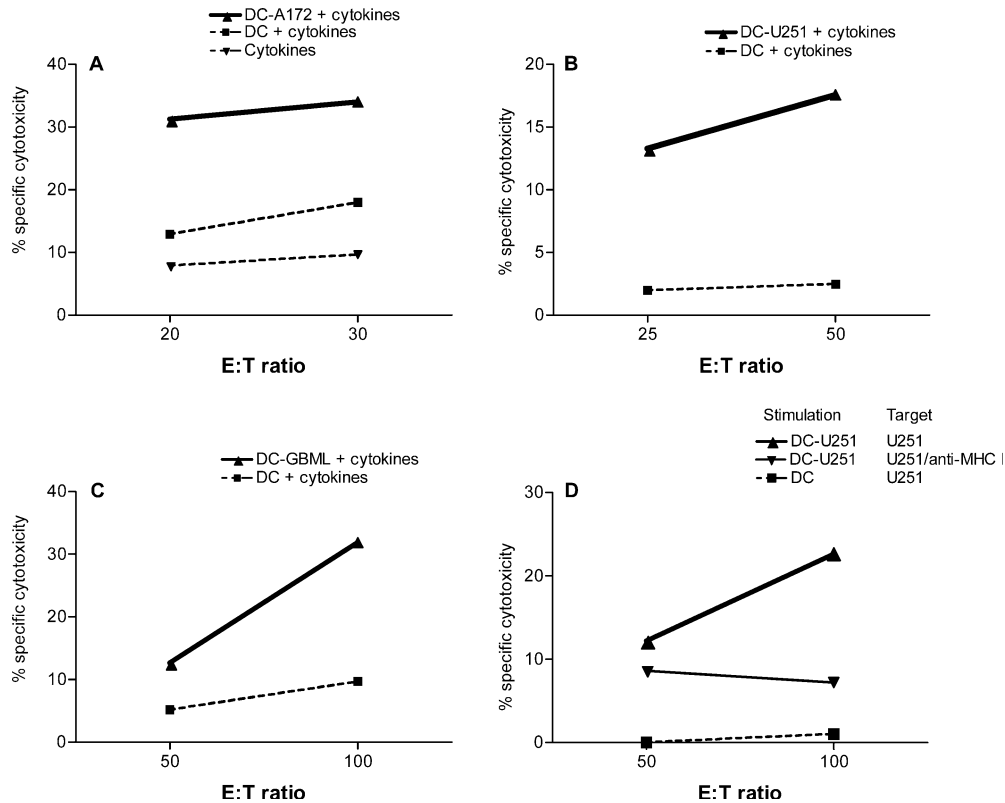


Fig. 5 CTL activity against tumor cells after stimulation by autologous DCs loaded with tumor lysate. T cells were cultured with autologous DCs loaded with tumor cell proteins, in the presence of rIL-6/rIL-12 and afterward in the presence of rIL-2/rIL-7, as described in “Material and methods.” T cells were cultured with unloaded DCs and cytokines or with cytokines alone as control conditions. Afterward, T cells were incubated at different E/T ratios with CTO-labeled tumor cells, during 3 h to evaluate cytotoxic activity. The binding of annexin V–FITC and uptake of 7-AAD by target cells was measured with FACS. The Y-axis shows the specific cytotoxicity that was calculated as described in “Material and methods.” **a** T cells and autologous DCs were derived from a normal donor who was compatible for HLA-A3 and HLA-B7 with the A172 cell line used for DC loading and as target. *DC-A172* DCs loaded with proteins derived from lysate of A172 tumor cells. **b** T cells and autologous DCs were derived from a patient who was compatible for HLA-A2 with the U251 cell line used for DC loading and as target. **c** T cells, autologous DCs, and autologous tumor cells were derived from a patient with malignant glioma. *DC-GBML* DCs loaded with proteins derived from the lysate of the tumor of the patient. **d** T cells and autologous DCs were derived from a donor who was compatible for HLA-A2 with the U251 cell line used for DC loading and as target. Prior to the cytotoxicity assay, the MHC class I molecules of the U251 cells were blocked by adding blocking anti-MHC class I mAb HB95

I context as internalized antigens can be transferred from endocytic compartments to the cytosol [36, 43]. In our experiments, the addition of a maturation cytokine cocktail resulted in a more pronounced colocalization of labeled tumor protein fragments and MHC class I and class II molecules. This finding is consistent with recent literature data showing that maturation of DCs improves peptide loading onto both MHC class II molecules [10] and class I molecules [22]. It is remarkable that

only a fraction of the labeled lysate is taken up, processed, and presented by the DCs on the membrane.

It should be noted that the antigenic properties of FITC-labeled proteins might differ. FITC contains an isothiocyanate group ($R1-N=C=S$) interacting under specific conditions with amine groups ($R2-NH_2$) on the amino acids. This results in the formation of a thiourea group ($R1-NH-C=S-NH-R2$) which is a stable and selective covalent binding. This binding makes FITC a preferred fluorophore to label proteins and peptides. Because the lysate consists of a mixture of undefined tumor antigens, the possible change of particular protein fragments after FITC binding can not be described in detail. These experiments were performed only to demonstrate the principle of loading, processing, and presenting of tumor proteins. For the generation of T-cell effector functions, native tumor lysate was used to load DCs.

During primary and secondary stimulation of the T cells, rIL-6 and rIL-12, and then rIL-2 and rIL-7, respectively, were added, as published by others [11, 25, 53]. Addition of rIL-12 shifts the T-cell response toward Th1 cytokine production, which is considered to be essential for the generation of a long-lasting specific immune response against tumor cells [17, 50]. Although rIL-6 might induce Th2 responses [15], it acts as a pro-inflammatory cytokine, and its use might be of particular interest because it overcomes putative functional effects of circulating $CD4^+CD25^+$ regulatory T cells on conventional T cells [39]. The use of rIL-2 and rIL-7 during secondary stimulation sustains primarily T-cell viability.

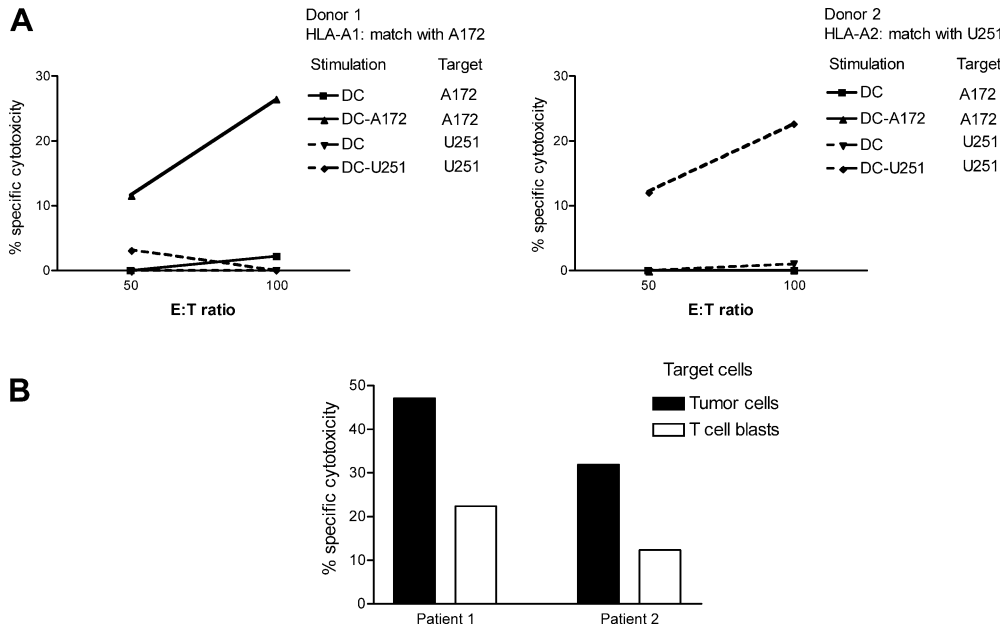


Fig. 6 Effector T cells have specific CTL activity against tumor cells. **a** T cells from two donors were stimulated with autologous loaded DCs in the presence of rIL-6/rIL-12 and rIL-2/rIL-7, respectively. As control, T cells were cocultured with unloaded DCs in the presence of cytokines. DC were loaded with lysates from GBM tumor cells, of which an HLA A allele was compatible with the donor cells, or from GBM tumor cells, of which an HLA A allele was not compatible. Cytotoxic activity was measured against the respective tumor cells. **b** T cells isolated from peripheral blood of two patients with malignant glioma were stimulated with autologous DCs loaded with tumor cell proteins from autologous tumor cells as described. Cytotoxic activity was measured against autologous tumor cells or autologous PHA-stimulated T-cell blasts. The E/T ratio was 100:1

We assessed the capacity of the loaded DCs for functional priming of T cells toward cytotoxic effector cells. Because GBM target cells could not be loaded in a reproducible way with chromium 51, we used a modified FCC assay [20, 32]. We demonstrated cytotoxic activity against autologous or partially MHC class I-matched GBM target cells when T cells had been stimulated in the presence of rIL-6/rIL-12 and rIL-2/rIL-7, with autologous DCs loaded with tumor cell proteins. The lack of cytotoxic activity when MHC class I molecules on the target cells were blocked, strongly suggests that at least a portion of the observed cytotoxic response is mediated by tumor antigen-specific T cells in a T-cell receptor-dependent manner. T cells cultured in the presence of rIL-6/rIL-12 and rIL-2/rIL-7 with or without unloaded DCs showed only weak activity in this cytotoxicity assay. Inherent to the use of tumor lysate as the source of tumor antigen, and the use of autologous or MHC class I compatible GBM cell lines as targets, it remains difficult to demonstrate target specificity. Indeed, the model relies on a mixture of undefined tumor antigens used for stimulation as well as undefined tumor antigens on the target cells [40]. This theoretically excludes any cell as “a perfect target control.” We decided to use autologous PHA-stimulated T-cell blasts as a control target as also done by

others [47]. Although T-cell blasts originate from a different germline, transformation to blasts might induce some “common antigenic epitopes” also expressed on GBM tumor cells. The differential cytotoxic activity against different but autologous target cells by T cells primed by autologous DCs loaded with tumor cell proteins clearly pointed to tumor-specific cytotoxic activity.

The most important finding in this paper is the feasibility of using whole tumor cell lysates as a source of antigens to induce tumor-specific T-cell responses. GBM are very heterogeneous tumors [5, 12, 48]. Although several tumor antigens have been described [12, 27, 31, 35, 38, 54, 57], a universally expressed common tumor antigen is lacking. Using a whole tumor cell lysate to load DCs circumvents this problem. Moreover, there are conceptual arguments to use a mixture of tumor cell proteins for loading DCs. The use of a single peptide as tumor antigen for vaccination can cause clonal selection of antigen-loss variants [42], unless the peptide is universally expressed and essential for tumor cell renewal, like apoptosis-inhibitory proteins [49]. An additional argument is the *in vivo* phenomenon of epitope (determinant) spreading shown in patients vaccinated with peptide-loaded DC vaccines [9]. This is consistent with the finding that multiple peptide epitopes are required for the induction of an effective antitumor immune response when MHC class I-binding peptides from tumor cells were used [41]. A further theoretical advantage of using whole tumor cell lysates is that loading DCs with unfractionated tumor material results in presentation of tumor antigens in the context of both MHC class I and MHC class II [36, 43]. By using confocal microscopy, we were able to demonstrate this particular phenomenon for protein fragments from GBM tumor cell lysates. Finally, cytosolic endogenous adjuvants were identified, which function as a danger signal to activate the DCs toward becoming full antigen-presenting cells [51].

However, the use of whole cell lysates to load DCs might result in an enhanced risk for the induction of autoimmunity. This phenomenon has not been reported yet for malignant gliomas in the animal models [24] or the clinical trials [14, 55, 58]. The induction of autoimmunity has been reported when T cells were loaded with melanoma-specific peptides [3].

We conclude that DCs can be loaded with proteins derived from lysates of GBM cells, and that these DCs can present antigens in an immunogenic way, which is effective in stimulating T cells for the generation of antitumoral cytotoxic effector functions. These essential data, which to our knowledge have not yet been elaborated in this way, support the use of autologous DCs loaded with tumor cell lysate as tumor vaccines to treat patients with malignant glioma in clinical phase I/II trials [14, 55, 58].

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