

## Antigen Loading of Dendritic Cells with Apoptotic Tumor Cell-Preparations is Superior to that Using Necrotic Cells or Tumor Lysates

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**Abstract.** *Background:* Loading of dendritic cells (DCs) with tumor cell (TC) preparations is an attractive method for vaccine preparation because the entire antigen repertoire of a tumor is processed and presented by the DCs, thus allowing the simultaneous stimulation of T-helper cells and cytotoxic T-lymphocytes. However, optimal loading conditions have still to be defined. *Materials and Methods:* DCs were pulsed either with tumor lysates, apoptotic or necrotic preparations of a breast cancer cell line and subsequently used to stimulate autologous T-lymphocytes. Antigen loading was quantified using immunofluorescent-based methods. *Results:* Four hours co-incubation of apoptotic TCs or tumor lysates with DCs undergoing maturation resulted in effective DC-loading. However, the DCs pulsed with apoptotic TCs were best in stimulating interferon- $\gamma$  (INF- $\gamma$ ) secretion as the effector function of autologous T-cells. *Conclusion:* Tumor lysates are in common use for DC-based vaccine manufacturing. However, our data indicate an advantage of apoptotic TC-preparations in regard to antigen loading effectiveness as well as the loaded DC's capacity to activate T-cells.

Dendritic cells (DCs) are the most potent antigen-presenting cells that are essential for initiating primary immune responses (1). Culture techniques and cytokines are available to allow the large scale *ex vivo* generation of DCs from peripheral blood monocytes (2). Thus, DC-based

vaccinations have been used in clinical studies to induce tumor-specific immunity as well as clinical responses in selected patients (3, 4). Strategies to introduce tumor antigens into DCs have included loading with either defined tumor antigens (peptides or proteins, transfer of tumor antigen-specific DNA or RNA) or whole tumor cell (TC) preparations.

In clinical trials one of the most common ways of generating DC-based vaccines has been pulsing them with human leukocyte antigen (HLA)-binding peptides, which are able to bind directly to HLA class I or class II molecules on the cell surface and are easy to manufacture (5). In general, these peptides represent ubiquitously expressed tumor-associated antigens (TAA) which induce immune responses applicable to most cancer patients with tumors expressing the appropriate antigen(s). It has been shown that peptide-pulsed DCs increase the peptide-specific cytotoxic T-lymphocytes (CTLs) in melanoma, renal cell carcinoma, breast and ovarian carcinoma patients (6-8). However, peptide-based vaccines are limited in their application, as they are restricted to the selected peptides and will only be efficacious for patients that express adequate HLA, which presents these particular antigenic peptides for recognition by the T-cells. Moreover, evidence has been presented that immunoselection for tumor variants that failed to express the appropriate antigens were responsible for recurrences after "monovalent" vaccination strategies applying only a few peptides (9, 10).

In contrast to peptide pulsing, using whole TC-preparations for DC-loading has the advantage that the entire repertoire of tumor antigens, including yet undefined TAA and individual mutations, are presented, making it less likely that antigen loss variants would escape. Furthermore, both HLA class I and II epitopes can be presented by autologous DCs, stimulating both CD8<sup>+</sup> and CD4<sup>+</sup> T-cells. The latter are of special importance because

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*Key Words:* Antigen-loading, dendritic cells, cancer vaccine, immunotherapy.

T-helper cells are believed to be necessary for long term CD8<sup>+</sup> T-cell memory and effective antitumor immune reactions (11-13). Defined HLA class II restricted epitopes for the induction of tumor-specific T-cell help are still rare. The disadvantages of whole TC preparations include the necessity to gain a sufficient number of autologous TCs without "contaminations" such as lymphocytes or benign tissue (14), the potential hazard of the induction of autoimmunity after professional presentation of non-tumor-antigens by DCs (15, 16), and the difficult validation of such an undefined vaccine. Controversy still exists regarding the most effective TC preparations for effective DC-loading as well as for stimulating immune priming (17-21). However, clinical trials have already been performed in patients with advanced metastatic disease using mainly tumor lysates obtained by freeze/thaw cycles or necrotic cells for DC pulsing (22-26).

In the current study three methods of preparation of breast cancer cells for uptake by immature monocyte derived DCs were investigated: apoptotic cells (generated by UV-B-irradiation); necrotic cell material (generated by heat-pretreatment) and cell lysates (generated by freeze/thaw cycles).

## Materials and Methods

*Tumor cell lines and tumor cell-derived antigen preparation.* The cell line KS was established in our laboratory from a malignant effusion of a breast cancer patient. The KS cells were maintained as adherent cultures in DMEM containing 10% fetal calf serum (FCS), 4 mM L-glutamine, and 1% penicillin/streptomycin (all Invitrogen, Paisley, UK). The CD80/Her-2/neu-cotransfected subline KS24.22 has been described previously and was maintained in the same way as the nontransfected parental counterpart, with the addition of G418 and Zeocin (Invitrogen), which were supplemented at 0.5 mg/ml and 0.25 mg/ml, respectively (27). The KS24.22-derived antigens were prepared using three different methods. i) Tumor lysates: The KS24.22 cells were detached by trypsin/EDTA solution (PAN Biotech, Aidenbach, Germany) and resuspended in culture medium (1x10<sup>7</sup> cells/ml). The TCs were subsequently treated with two rapid freeze/thaw cycles (-140°C / 21°C); one cycle consisted of 10 sec in liquid nitrogen followed by 10 min at room temperature (TC<sup>lysate</sup>). ii) Heat-treatment: The KS24.22 cells (5x10<sup>6</sup>) were incubated for 2.5, 5, 7.5 or 10 min at 58°C in a volume of 0.5 ml in eppendorf cups using a water-bath. Subsequently, the cells were transferred to culture flasks and incubated for 24 h in 10 ml culture medium (37°C, 5% CO<sub>2</sub>). Nonadherent TCs were harvested, washed, and further analyzed (TC<sup>58°C</sup>). iii) UV-treatment: A 5 ml KS24.22 cell suspension was added to a 100 mm<sup>2</sup> culture dish (Becton Dickinson, Franklin Lakes, NY, USA) and exposed to UV-B light (312 nm, M&S Laborgeräte GmbH, Wiesloch, Germany) for 1, 5, 10, or 15 min (450 µW/cm<sup>2</sup>). After exposure, the cells were washed in phosphate-buffered saline (PBS) and further cultured in DMEM (37°C, 5% CO<sub>2</sub>) to allow apoptosis to occur. After 24 h, the nonadherent portion was collected, washed, and used for additional experiments (TC<sup>UV</sup>).

*Detection of apoptosis and necrosis.* The differently pretreated TCs were assessed for induction of apoptosis as well as necrosis by using Annexin-staining (early apoptosis) and TUNEL assay (late apoptosis). Staining with Annexin V-Fluos was performed according to the manufacturer's protocol (Roche, Mannheim, Germany) and combined with propidium iodide (PI)-staining. Briefly, 1x10<sup>6</sup> TCs were washed and resuspended in 50 µl 10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 5 mM CaCl<sub>2</sub> containing Annexin V-Fluos (1:50 stock-dilution) as well as PI (20 µg/ml). After staining for 15 min at 15-25°C, cells were analyzed using an EPICS<sup>®</sup> XL-MCL cytometer (Coulter Electronics, Hialeah, Finland) with logarithmic amplification (3log scale). The results were processed using the appropriate software (Coulter). For analysis of caspase 3/7 induction the Apo-One™ Homogenous Caspase-3/7 Assay (Promega, Madison, USA) was used according to the manufacturer's instructions. Briefly, the TCs were either UV-irradiated, heat-pretreated or subjected to freeze/thaw cycles as described above. After incubation for 4, 24, or 48 h (37°C, 5% CO<sub>2</sub>), the TCs were harvested, washed and distributed into a 96-well plate (1x10<sup>5</sup>/well) and the fluorogenic caspase 3/7 substrate rhodamine 110 was added. After cell lysis, active caspases cleave a proluminescent substrate, thereby liberating free aminoluciferin, which is consumed by luciferase. Fluorescence was measured as Relative Light Units (RLU) on a multilabel counter (Wallac Victor™ 1420, Wallac Oy, Turku, SF) after an incubation period of 18 h. In the TUNEL assays, 1-2x10<sup>6</sup> cells were analyzed following the instructions provided by the manufacturer (Apoptosis Detection System, Fluorescein, Promega). Incubation with TdT and fluorescein-labeled d-UTP provided visualization of DNA fragmentation by flow cytometry.

### *Dendritic cells.*

*Generation of DCs.* Concentrated blood samples ("buffy coats") of healthy donors were obtained from the Institute of Immunology with Blood Bank, University of Heidelberg, Germany, according to the institutional guidelines. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque (Seromed Biochrome KG, Berlin, Germany) density centrifugation. Immature DCs were generated from monocytes enriched from the PBMCs by plastic-adherence in culture flasks (Nunc, Roskilde, Denmark) and maintained in X-Vivo 15 medium (BioWhittaker, Walkersville, Maryland, USA) supplemented with 1000 U/ml IL-4 (R&D-Systems, Wiesbaden, Germany) and 1000 U/ml GM-CSF (Leukomax<sup>®</sup>, Novartis, Basel, Switzerland) (DC-medium). At day 7, the immature DCs were collected and cultured in fresh DC-medium, further supplemented with TNF-α (10 ng/ml), IL-1β (10 ng/ml), IL-6 (1000 U/ml) (all R&D Systems), and PGE<sub>2</sub> (1 µg/ml, Minprostin<sup>®</sup>, Pharmacia & Upjohn, Erlangen, Germany) to induce maturation (28). At the same time, DCs were loaded with TC-preparations as described below. The cells were harvested after 2 days and used for further experiments.

*Phenotypic analysis of DCs.* Phenotypisation of the DCs was performed after blocking with 5% Endobulin Immuno<sup>®</sup> (Baxter, München, Germany) in FACS-buffer (PBS, 10% FCS, 0.1% NaN<sub>3</sub>) using the following either PE-, or FITC-conjugated monoclonal antibodies: IgG1-PE, IgG1-FITC, IgG1-PC5 as isotype controls, anti-CD86, anti-CD83, anti-HLA-ABC, anti-HLA-DR (Coulter Immunotech, Marseille, France), and anti-CD1a (BD Pharmingen, Heidelberg, Germany). Cells were fixed using 1% formaldehyde and analyzed on an EPICS<sup>®</sup> XL-MCL flow cytometer (Coulter Electronics).

**Cryopreservation of DCs.** The DCs were harvested and suspended at a concentration of  $2 \times 10^6$ /ml in 10% glucose, X-Vivo 15. 0.5 ml cell suspension was transferred to a 1.8 ml cryotube (Nunc) and stored for 10 min on ice. An equal volume of cryo-medium (20% DMSO, human serum (HS), 4°C) was added and probes were frozen at  $-1^\circ\text{C}/\text{min}$  in a cryo/freezing-box (Nalgene, Roskilde, Denmark) to  $-80^\circ\text{C}$ . After 24 h, cryoconserved cells were transferred to liquid nitrogen for further storage. The cells were kept frozen for 1 week at minimum. For thawing, pre-warmed media was transferred to the cryotubes. The thawed cells were gradually transferred to DC-medium and washed twice.

**Antigen-loading onto dendritic cells.** The day 7 DC cultures were loaded with KS24.22-derived antigens from the three different preparation methods: tumor lysates ( $\text{TC}^{\text{lysate}}$ ), heat-treatment ( $\text{TC}^{58^\circ\text{C}}$ ) or UV-treatment ( $\text{TC}^{\text{UV}}$ ). The DCs ( $1 \times 10^6$ ) were co-incubated in 6-well plates at a ratio of 1 DC to 1 KS24.22 cell equivalent in a volume of 3 ml DC-medium. After 24 h, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and PGE $_2$  were added and the cells were incubated for an additional 4-48 h at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ .

**Assessment of antigen-loading (phagocytosis assay).** To assess the uptake of KS24.22-derived antigens by the DCs, the TCs were first labeled with CellTracker™ green fluorescent dye 5-chloromethylfluorescein diacetate (CMFDA), (Molecular Probes, Leiden, The Netherlands). To this end, 0.25  $\mu\text{l}$  CMFDA (10 mM in DMSO) was added to  $1 \times 10^7$ /ml KS24.22 cells and incubated for 30 min at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . The labeled TCs were washed twice in culture medium and then used for antigen preparation and DC-loading. After coculture of KS24.22-derived antigens with the DCs, the cells were subjected to flow cytometry as well as immunofluorescence microscopy. For FACS analysis, DCs were identified using an anti-CD86 PE-conjugated monoclonal antibody, a marker absent from the KS24.22 cells. PE-labeled cells give a signal in the FL-2 channel whereas CMFDA-labeled cell preparations appear in the FL-1 channel. Thus, any DC which took up tumor cell fragments would be detected in both channels as a double-labeled cell. In parallel, cytopins of  $1\text{-}2 \times 10^5$  loaded DCs were prepared, air dried at RT for 24 h in the dark and subsequently analyzed using an immunofluorescence microscope (U-ULH Olympus Optical Co., Ltd, Japan) to differentiate between cells that only adhered to each other and the DCs which had taken up tumor material.

**Immunostimulatory function of dendritic cells.** After antigen-pulse and maturation, the DCs ("stimulators") were added to purified T-cells (>97% CD3 $^+$  cells after negative magnetic cell sorting using a pan T-cell selection kit, Miltenyi, Bergisch Gladbach, Germany) of the same donor ("autologous responders") at a ratio of 1:10 in 96-well plates. Unpulsed DCs were used as controls. After 3 days of coculture, IL-2 (30 U/ml, Cetus GmbH, Solothurn, Switzerland) was added to the T-cell medium (RPMI 1640, 2 mM L-glutamine, 1% penicillin/streptomycin (all Invitrogen), 10% human AB serum (ccpro, Neustadt, Germany)). The T-lymphocytes were then restimulated twice by the addition of the appropriate antigen-pulsed DCs on a weekly basis. Seventy-two hours after the second restimulation, the culture supernatants were harvested and analyzed for IFN- $\gamma$  secretion of the activated T-cells by a standard ELISA according to the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden).

## Results

**Characterization of whole tumor cell preparations.** It was determined that 24 hours incubation after 1-10 min UV-irradiation was optimal for inducing apoptosis of the TCs, with approximately 80% of TCs showing nuclear DNA-fragmentation as a sign of late apoptosis by TUNEL assay (Figure 1A). Four hours incubation time was not sufficient to detect DNA-fragmentation and after 48 h the apoptotic cell fraction had increased just slightly (not shown). This was confirmed by the activity of caspase-3, one of the most important "death proteases" triggering the cleavage of numerous key proteins leading to the controlled suicide of cells (29). Caspase-3 activity in the KS24.22 cells was highest after 1 min UV-exposure and stable for 24 h (Figure 1B). In contrast, freeze/thaw cycles ( $\text{TC}^{\text{lysate}}$ ) or heat-pretreatment of the TCs ( $\text{TC}^{58^\circ\text{C}}$ ) resulted in neither DNA-fragmentation nor caspase-3 activation (Figure 1A-D). The  $\text{TC}^{58^\circ\text{C}}$  preparations predominately contained necrotic cells as shown by Annexin/PI double-staining, further verified by trypan blue staining (data not shown).

**DC-loading and phagocytosis assay.** The flow cytometry results of the DCs cocultured with the KS24.22-derived antigens are shown in Figure 2A. Within 4 h of coculture, tumor antigens could be detected in the DCs and assessed as the percentage of double-fluorescent cells; thus 7.1% of the DCs cocultured with untreated tumor cells (DC+TC), 75.9% with  $\text{TC}^{\text{UV}}$ , 8.2% with  $\text{TC}^{58^\circ\text{C}}$  and 73.1% with  $\text{TC}^{\text{lysate}}$  were tumor antigen-positive. To confirm the presence of double-fluorescence within antigen-pulsed DCs, cytopins were prepared in parallel and analyzed by confocal microscopy to visualize antigen uptake. Figure 2B shows an example of DCs pulsed with  $\text{TC}^{\text{UV}}$ . After 4 h, DCs had phagocytosed the apoptotic TCs and within 24 h, tumor-derived antigens had been processed and were found in the vesicles. This indicated that the double-positive labeled cells were not due to TC material simply adhering to the DC surface. Quantification of antigen-uptake from apoptotic ( $\text{TC}^{\text{UV}}$ ) and necrotic ( $\text{TC}^{58^\circ\text{C}}$ ) TCs using either FACS or immunofluorescence microscopy demonstrated a good correlation regarding the frequency of antigen-loaded, double-stained DCs (data not shown).

**Apoptotic tumor cells are more efficient in DC-loading compared to necrotic tumor cell-preparations.** To compare and optimize antigen-loading efficiency, DCs were cocultured with different TC formulations in a DC-to-TC-equivalent ratio of 1:1 for 4, 24, and 48 h, respectively. Antigen uptake was quantified by flow cytometry. The  $\text{TC}^{\text{UV}}$  preparations were obtained after irradiation times of between 1 and 15 min. The best DC-loading was demonstrated after 4 h of cocultivation with  $\text{TC}^{\text{UV}}$  irradiated for 1 min (76% CD83 $^+$ /CMFDA $^+$  DCs) compared to untreated TCs as the antigen source (7%) (Figure 3A). The

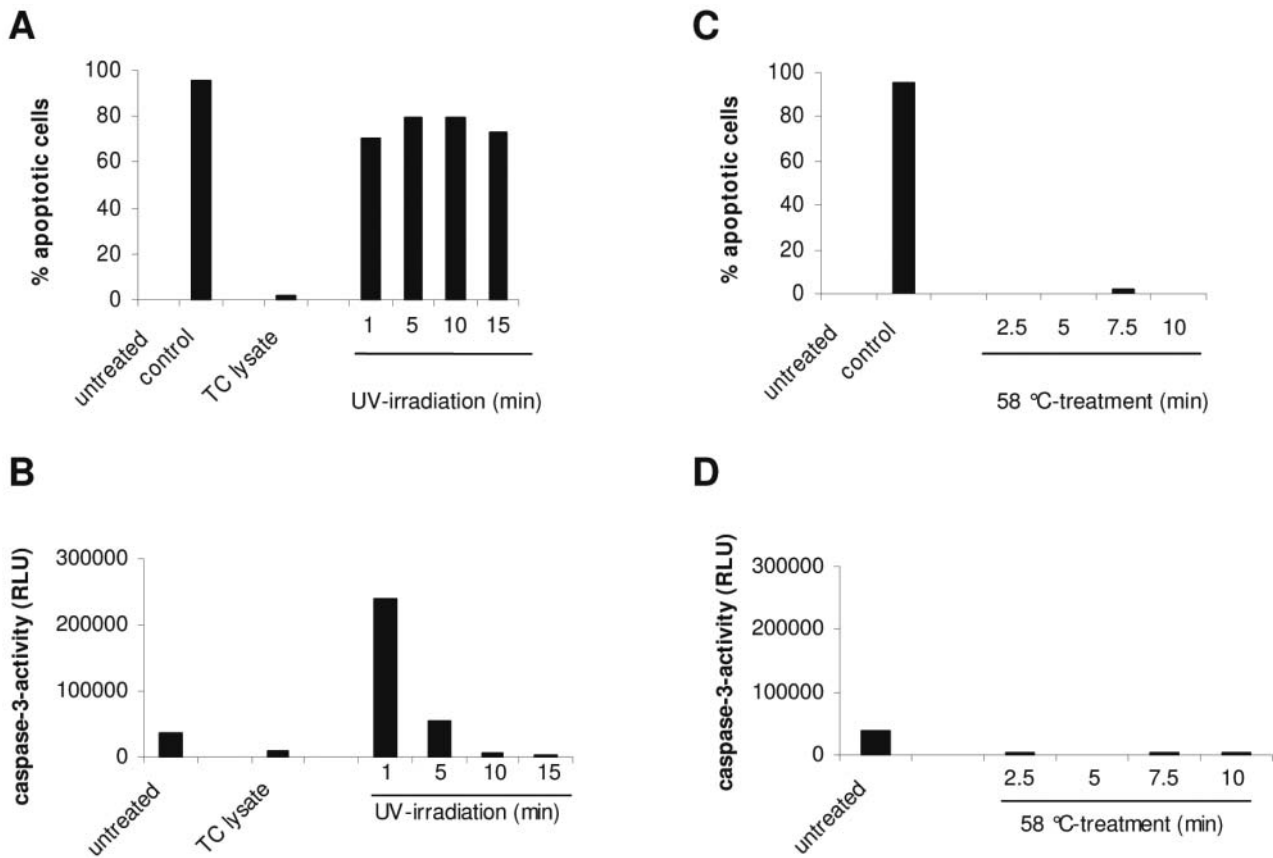


Figure 1. UV-irradiation induces apoptosis in a breast cancer cell line. Tumor lysate preparations, ( $TC^{lysate}$ ), UV-light- ( $TC^{UV}$ ), or heat-exposed TCs ( $TC^{58^{\circ}C}$ ) were examined 24 h after treatment for the induction of apoptosis. (A, C) In TUNEL-assays, the proportion of TCs undergoing DNA-fragmentation as a sign of late apoptosis was detected by flow cytometry. Untreated TCs were analysed in parallel and did not show any DNA-fragmentation. DNase I-treated cells were used as positive control. (B, D) Caspase-3 activity of pretreated TCs was quantified by a chemiluminescence-based assay. The generated luminescent signal (RLU) is proportional to caspase activity. The data are representative of three independent experiments.

$TC^{UV}$  (1 min) preparations contained the highest proportion of apoptotic cells, as shown using TUNEL assay (Figure 1B). Longer UV-exposure as well as prolonged antigen-pulse did not increase antigen uptake, probably due to the growing portion of necrotic TC material over time. The uptake of "untreated" TCs also increased over 48 h (7% to 51%) which was related to the appearance of apoptotic cells (data not shown). The  $TC^{lysate}$  was as efficiently phagocytosed as apoptotic TCs within 4 h. Here, the population of  $CD83^{+}/CMFDA^{+}$  DCs clearly diminished over time, probably due to intracellular processing. Figure 3B shows that CMFDA-labeled necrotic cell material ( $TC^{58^{\circ}C}$ ) was only phagocytosed by 5-11.7% of the total DCs. Neither the duration of heat-treatment during  $TC^{58^{\circ}C}$  preparation nor the DC/ $TC^{58^{\circ}C}$  coincubation time had a positive input on antigen-uptake. To determine the capacity of cryopreserved DCs for antigen-uptake, the same set of experiments was performed using immature DCs, frozen on day 7, thawed and recultured for 24

h in DC medium and further supplemented with cytokines as described in material and methods. The antigen uptake by cryopreserved DCs was clearly reduced by up to 50% for all TC preparations (4 h coincubation) (Figure 3C, D). In contrast to fresh DC-preparations, prolonged coincubation of thawed DCs with apoptotic TCs ( $TC^{UV}$ ) had a positive impact on phagocytosis (Figure 3C).

*DCs loaded with apoptotic tumor cells are superior in T-cell activation.* In order to know whether different TC preparations for DC-pulsing yielded comparable levels of T-cell effector capacities stimulation experiments were performed. DCs optimized for loading with KS24.22-derived antigens by the three methods described above were used for the induction of T-cell  $INF-\gamma$  secretion in an autologous setting. Figure 4 summarizes data of four independent experiments, using DCs and  $CD3^{+}$  T-lymphocytes of the same donors, respectively. All approaches result in  $INF-\gamma$

production. However, using DC/TC<sup>58°C</sup> (701.2±59.8 ng/ml IFN- $\gamma$ ) or DC/TC<sup>lysate</sup> (1078.4±430 ng/ml) as the stimulator cells did not result in notably higher IFN- $\gamma$  secretion than the control unpulsed DCs (944.8±617.6 ng/ml). In contrast, DCs which were pulsed with apoptotic KS24.22 cells (TC<sup>UV</sup>) stimulated a potent T-cell response, giving a mean±SD of 2531.2±295.8 ng/ml IFN- $\gamma$ .

## Discussion

Only a few tumor rejection antigens have been identified for the treatment of breast cancer and other cancer types making the possibility of whole TC-based vaccines an attractive option. The parallel application of adjuvants is a prerequisite for immunostimulation because TCs are generally considered to be poorly immunogenic due, for instance, to reduced expression of costimulating molecules and alterations in the HLA class I and II antigen processing pathways. In contrast, DCs are the most potent antigen-presenting cells and have been widely used as so called "nature's adjuvant" for the induction of antitumoral immunity. Initially, polyethylene glycol- or electro-fusions of DCs and TCs were the most advanced technology, but, several technical problems such as insufficient cell recovery and lack of appropriate quality control of fusion products restricted a broadened clinical application (30).

The present study of three methods of preparation of breast cancer cells has demonstrated that short-time UV-B-irradiation induced TC apoptosis while heat-pretreatment and repetitive freeze/thaw cycles generated necrotic cells. In accordance with the danger model of immunity first described by Matzinger (31), the DCs were able to phagocytose both, apoptotic and necrotic TCs, confirming other data (32). However, by using flow cytometry and fluorescence microscopy to compare and quantify antigen uptake, the best results were achieved by using apoptotic TCs as the antigen source. After 24 h, tumor-derived antigens could be seen in the vesicles of the DCs, indicating activated processing pathways. In line with other studies, the proportion of double-stained DCs diminished over time after antigen-uptake, probably due to intracellular antigen processing. On the other hand, an increase in antigen uptake using untreated TCs was seen, attributed to TC death *via* necrosis with prolonged incubation times (32).

Both, lysate and heat-pretreated TCs showed Annexin/PI staining, however, after 58°C pretreatment the TCs were generally intact while incorporating trypan blue, whereas the freeze/thaw cycles resulted in fragmentation of cells and presumably organelles and nuclei. The distinction between sources of necrotic TCs became apparent in the phagocytosis assay, where TC<sup>lysate</sup> were also phagocytosed as were apoptotic TC<sup>UV</sup> in contrast to only small amounts of TC<sup>58°C</sup>. This could be due to

different maturation and maybe other factors released as a consequence of different types of TC death (33). Controversy exists over whether necrotic or apoptotic TCs are most advantageous in stimulating tumor-reacting T-cells (18, 34, 35). The failure of DCs coincubated with TC<sup>58°C</sup> to stimulate the T-cells was in line with the absence of proper antigen uptake. However, despite demonstrable antigen uptake, the DCs loaded with necrotic TC<sup>lysate</sup> did not induce T-cell IFN- $\gamma$  secretion. In our case, the initiation of DC maturation indicated by CD86 up-regulation using the apoptotic TC<sup>UV</sup> was more reliable than employing necrotic TC<sup>lysate</sup> preparations (data not shown). Several investigators have reported that bystander apoptosis triggers DC maturation and antigen-presenting functions, thereby promoting the stimulation of CTL (36, 37). The failure of the necrotic TC material to stimulate the DCs could have been related to the TC lines used and their individual pattern of liberated heat shock proteins during necrosis or the lack of additional bacterial lipopolysaccharides during DC maturation, as suggested by others (35). Additionally, the selection of either HLA class I or II antigen restriction has been shown to be related to the size of internalized material. Huge phagocytosed particles such as apoptotic bodies are found early in discrete well-defined vacuoles and have direct access to transporters associated with antigen processing (TAP)-transmitted transport followed by MHC class I presentation, a process which is 300 times more efficient than using pre-processed peptides (32, 38, 39). Small necrotic cell particles have been shown to be processed *via* the endosomal compartment which leads predominately to HLA class II restricted antigen presentation and subsequent CD4<sup>+</sup> T-cell activation (35, 40).

For clinical application, cryopreservation of DCs has certain advantages. Our data demonstrated that thawed immature DCs were clearly unsuitable for subsequent antigen-loading using whole TC preparations, probably due to the reduction of energy-dependent cellular processes and membrane damage after cryopreservation/thawing procedures (41). Interestingly, in contrast to "fresh" DCs- prolonged coincubation time had a positive impact on antigen-uptake using apoptotic TC<sup>UV</sup>. Other groups have demonstrated the practicability of cryopreserved DCs as a "ready to use" vaccine loaded and matured before freezing without loss of stimulatory capacity. However, the recovery of cryopreserved DC is reduced and a cell loss up to 40% should be expected (21).

In summary, these findings may be helpful for the design of vaccination trials based on whole TC preparations. Despite continuing controversy regarding whether necrotic or apoptotic TC preparations are best for DC-based vaccination trials, in our case the use of UV-irradiated apoptotic TCs was clearly superior for generating stimulatory DCs.

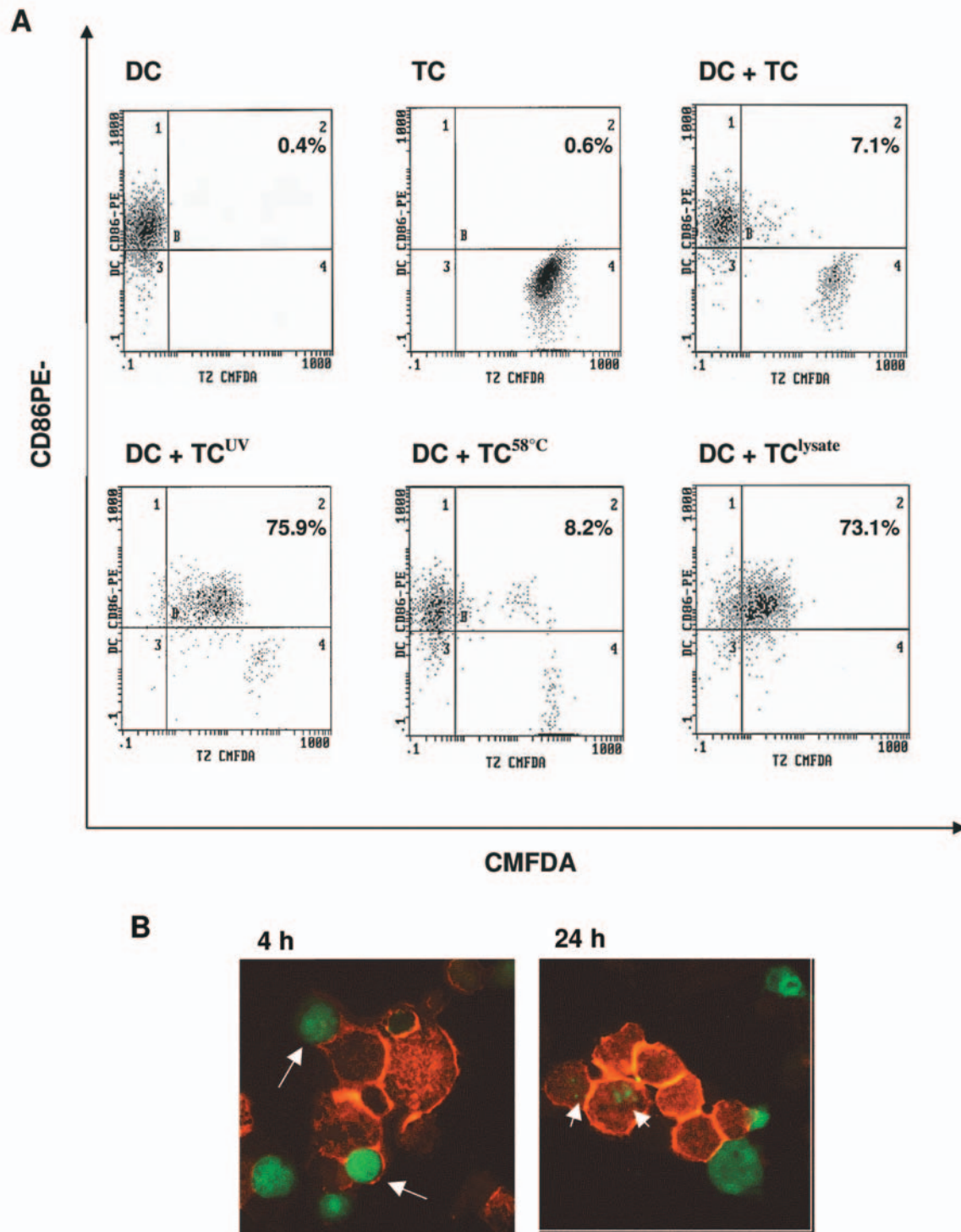


Figure 2. Immunofluorescence-based methods to quantify tumor cell-derived antigen-uptake of dendritic cells. For immunofluorescence-based phagocytosis assay, TCs were labeled with the green fluorescent dye CMFDA before antigen-preparation by UV-irradiation (1 min) ( $TC^{UV}$ ), heat-pretreatment (5 min) ( $TC^{58^{\circ}C}$ ) or lysate-fabrication ( $TC^{lysate}$ ). Untreated TCs as well as the three different TC-formulations were coincubated for 4 h with DCs undergoing maturation. Before analysis, DCs were labeled with anti-CD86-PE. (A) For FACS analysis, DCs were identified by CD86-PE-staining (FL-2) whereas CMFDA-labeled TC preparations appear green (FL-1). DCs which took up TC-derived antigens are detected as double-positive cells (top right quadrant). (B) Immunofluorescence microscopy of DCs 4 and 24 h after coincubation with  $TC^{UV}$  (1 min)-preparations. After 4 h, green fluorescent TCs were phagocytosed by DCs shown in red. Within 24 h, tumor-derived antigens had been processed and could be found in vesicles of DCs, indicated by the

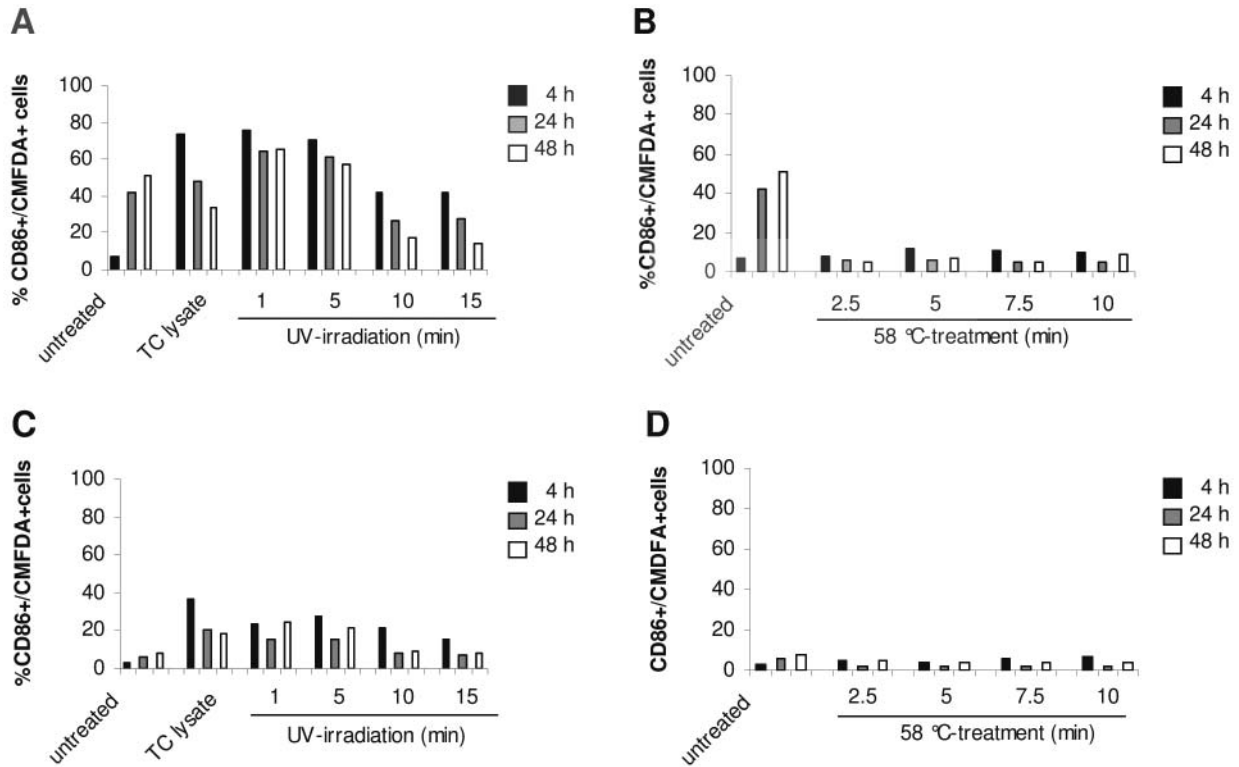


Figure 3. Comparison of antigen-uptake by dendritic cells pulsed with apoptotic versus necrotic tumor cell preparations. For immunofluorescence-based phagocytosis assays TCs were labeled with the green fluorescent dye CMFDA before antigen-preparation by UV-irradiation (1-15 min) ( $TC^{UV}$ ), heat-pretreatment (2.5-10 min) ( $TC^{58^{\circ}C}$ ) or lysate-fabrication ( $TC^{lysate}$ ). Untreated TCs as well as the three different TC formulations were coincubated for 4 h with DCs undergoing maturation. DCs were labeled using an anti-CD86-PE antibody. (A, B) "Fresh" DCs were cocultured with untreated TCs or different TC formulations for 4, 24, and 48 h. (C, D) Cryopreserved DCs were thawed and analysed for antigen-uptake using the same experimental setting. Compared to fresh DCs, antigen-uptake was clearly reduced in all cases. The data are representative of three independent experiments.

### Acknowledgements

We thank Susanne Stumm for her support, input and helpful discussions. We further acknowledge the expert technical assistance of Jutta Funk.

This study was partially supported by grants from the "Deutsche Krebshilfe" (70-2917) and the "AKF-Programm" of the University of Tübingen (77-1-0) (BG).

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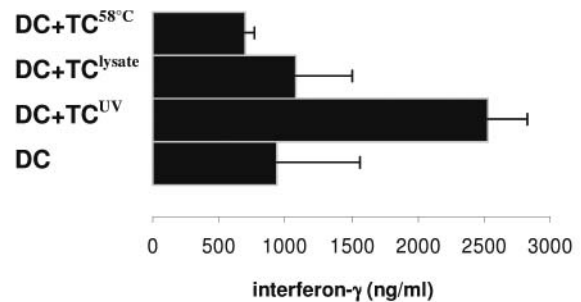


Figure 4. Dendritic cells loaded with apoptotic tumor cells induce potent T-cell reactions. The capacity of loaded and matured DCs to stimulate autologous  $CD3^{+}$  T-cells was tested by IFN- $\gamma$  secretion in response to short-term T-cell restimulation. The following TC preparations and loading conditions were chosen: lysate-preparation, 1 min UV-B-exposure, and 2.5 min heat-pretreatment. For DC-loading, each TC formulation was coincubated for 4 h at a ratio of one DC to one TC equivalent (see Materials and Methods). Loaded ( $DC/TC^{UV}$ ,  $DC/TC^{58^{\circ}C}$ ,  $DC/TC^{lysate}$ ) as well as unloaded DCs were used to stimulate  $CD3^{+}$  T-cells of the same donor for 14 days. Seventy-two hours after the last stimulation, culture supernatants were analysed for IFN- $\gamma$  concentration by ELISA. Mean values of four independent experiments using four different healthy donors are given  $\pm$ SD.

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*Received January 25, 2007*

*Revised April 2, 2007*

*Accepted May 4, 2007*