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Characterization of cells prepared by dendritic cell-tumor cell fusion

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Abstract

Dendritic cells (DCs) are professional antigen-presenting cells currently being discussed as a potent tool for antitumor vaccination strategies. The approach consisting of the *in vitro* generation of DC-tumor cell hybrids may be advantageous for individualized vaccines since there is no need for the determination of MHC-restricted tumor-associated antigens recognized by T cells. As recent vaccination studies gave varying results, we tested the impact of the fusion treatment on the cells used. Polyethylene glycol-induced fusion, as well as electrofusion, proved to be suitable for generating hybrid cells although at a low frequency. Of note, both methods also gave rise to DCs having phagocytosed apoptotic tumor cells. The expression of surface molecules relevant for specific T cell stimulation was not altered by the fusion procedure and the DCs were still functionally active as demonstrated by the secretion of IL-12 and the uptake of antigen. The cells were able to induce a tumor-specific T cell response *in vitro* and therefore deserve further investigation as potent tools for immunotherapy trials.

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells capable of initiating specific primary immune responses, including the activation of tumor-reactive cytotoxic T-cells (CTLs) (1, 2). Various tumor vaccination strategies have been developed based on the loading of DCs with tumor-associated antigens (TAAs) (3, 4). DC loading can be achieved either by pulsing with peptides and proteins or by gene transduction (5, 6). Since our knowledge of tumor-specific antigens is limited, some approaches involve loading DCs with apoptotic tumor cells or cell lysate (7, 8).

Another promising alternative consists in the fusion of DCs with tumor cells (9, 10). This approach is based on the idea that multiple TAAs are endogenously processed and presented by MHC class I molecules, thereby stimulating tumor-specific CTLs (11). Several mouse models and vaccination studies have shown regression of established tumors after hybrid cell application (9, 12, 13, 14, 15, 16).

In the present study, the cell populations generated either by polyethylene glycol (PEG) or electrofusion of DCs and tumor cells were characterized. We show that fusion of these two cell types is feasible. In addition, we show that the resulting cell population retains the functional capability of antigen-presenting cells essential for the induction of antigen-specific immune responses. Our data support the idea that the fusion of dendritic and tumor cells provides a promising approach for the immunotherapy of cancer.

Results

Comparison of hybrid cells generated by PEG fusion and electrofusion

We first analyzed the generation of hybrid cells by different fusion methods. Using the vital cell dyes CMTMR and CMFDA, hybrid cells could be detected as dual-fluorescent cells by fluorescence microscopy after either PEG fusion (Figure 1, A and B) or electrofusion (data not shown). To exclude diffusion of the dye, a second set of experiments was performed: Dendritic and tumor cells were first subjected to the fusion procedure and then stained with DC-specific and melanoma-specific mAbs. Using digital confocal microscopy, hybrid cells were shown to express DC-specific, as well as tumor cell-specific, surface antigens (Figure 1C).

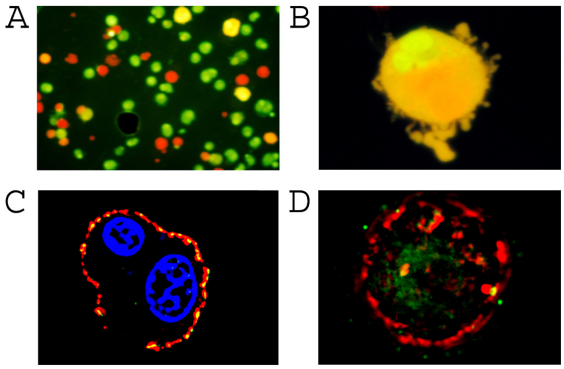

Figure 1. Hybrid cell generation. (A) CMTMR-stained DCs and CMFDA-stained melanoma (Mel Im) cells were fused using 50% PEG. Using a filter set appropriate for both emission wavelengths, DCs were detected as red cells, melanoma cells as green cells and hybrid cells as yellow cells (100x). More tumor cells than DCs are visible in different panels due to the unequal distribution of cells. (B) Hybrid cell consisting of a dendritic cell (CMTMR staining in red) and a Mel Im melanoma cell (CMFDA staining in green) (600x). Cells were prepared and analyzed as in 1A. (C) Analysis of hybrid cells using digital confocal fluorescence microscopy. After fusion of cells with 50% PEG without prior dye staining, cells were immunostained with anti-CD36-FITC/ anti-CD11c-FITC mAbs, the melanoma-specific anti-Mel1 antibody and the Rhodamine Red secondary antibody. Nuclear counterstaining was performed using Hoechst 33342. Hybrid cells were detected as dual-fluorescent cells with two nuclei. A representative image of a hybrid cell is shown (1000x). (D) To demonstrate phagocytosis of tumor cells, melanoma cells (Mel Im) were stained with PKH2-GL, admixed to DCs and treated with PEG. Samples were then stained with anti-CD36, anti-CD1a, and Rhodamine Red secondary antibody to label DCs. Analysis was performed using a digital confocal fluorescence microscope. A representative image is shown (1000x).

In addition to hybrid cells and cell aggregates, we found DCs having phagocytosed tumor cell material. We had to use a different staining method to clearly discern these events. PKH2-GL-stained melanoma cells and unstained DCs were treated with PEG and stained afterwards with mAb in order to label the surface of the DCs. Analysis by digital confocal fluorescence microscopy revealed that some of the DCs did indeed ingest tumor cells when subjected to the fusion procedure (Figure 1D).

Cellular events can be precisely defined by digital confocal microscopy. However the method is too slow for the quantitative evaluation of many cells. Therefore the efficiency of cell fusion was investigated quantitatively by flow cytometry using vital cell staining. One to three percent dual-fluorescent cells were detected when following PEG fusion of DCs with a melanoma cell line (Figure 2A) or non-melanoma cells lines (data not shown). For electrofusion, a direct current between 50 and 100 V per cm and a pulse of 25 µF - 1000 V/cm were selected based on pilot experiments, resulting in a cell survival of 50-95%. Figure 2B shows one representative example resulting in 7-14% dual-fluorescent cells; in some experiments up to 17% dual fluorescent cells could be obtained. Figure 2C shows that electrofusion of DCs with primary melanoma cells resulted in 15 to 20% dual-fluorescent cells. Similar results were obtained using primary cells from renal cell carcinoma (data not shown). Coculture controls were set up to determine the background rate of mere aggregation and phagocytosis. In PEG fusion control experiments, 0.5 to 1.5% dual-fluorescent cells could be detected, while electrofusion control experiments reached 0.5 to 5%. This variation might result from the treatment of cells for electrofusion, a more toxic treatment which was necessary to eliminate all the contaminant salt prior to the electrical pulse. This may lead to additional cell debris and a stronger spontaneous formation of cell clusters, a phenomenon that always has to be taken into account when comparing fusion efficiencies.

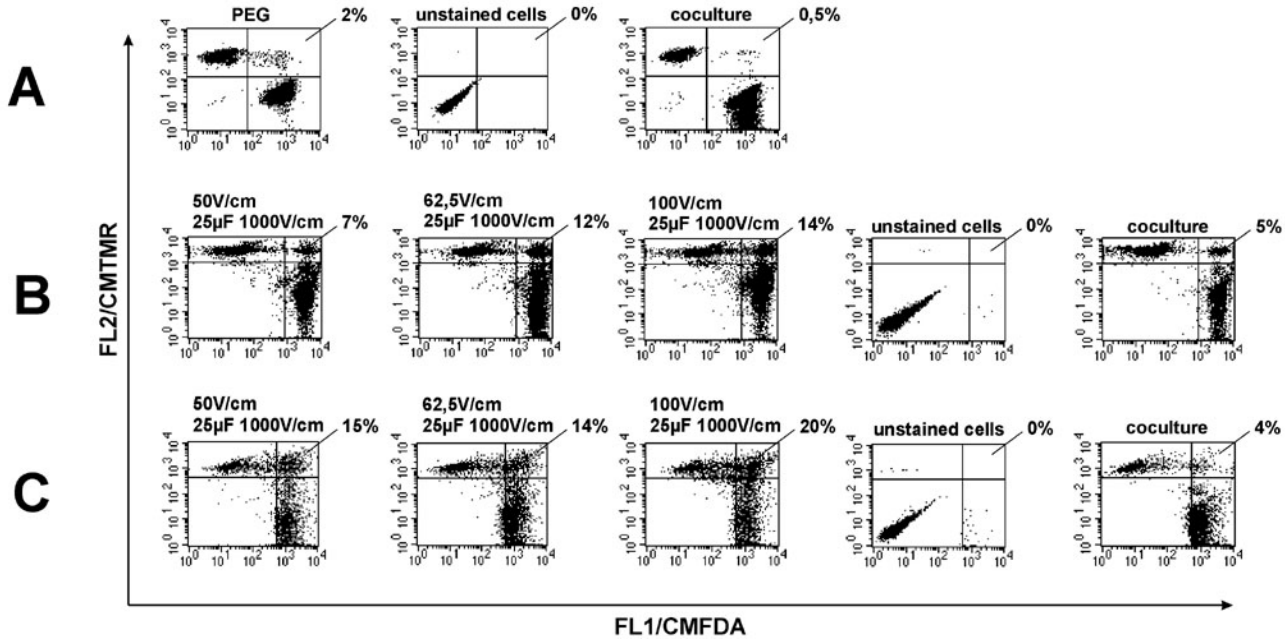

Figure 2. Quantification of fusion by flow cytometry. (A) CMTMR-stained DCs and CMFDA-stained melanoma (Mel Im) cells were fused using 50% PEG. Three hours after fusion, samples were analyzed by flow cytometry. The percentage of dual-fluorescent cells is indicated for each sample. As controls, cells cocultured for 3 hours without treatment and unstained cells were analyzed. (B) CMTMR-stained DCs and CMFDA-stained melanoma (Mel Im) cells were electrofused using different parameters. A constant current of 50, 62.5 or 100 V per cm was applied to the cells prior to delivery of a pulse of 25 µF - 1000 V/cm. All samples were analyzed as described in 2A. (C) CMTMR-stained DCs and CMFDA-stained primary melanoma cells were electrofused using different parameters as indicated in 2B.

Phenotypic and functional characterization of DCs after fusion with tumor cells

Next, we were interested in determining if the fusion procedure affected the phenotype and function of the DCs. For four hours after fusion, cell viability was between 50 and 95% as seen by trypan blue exclusion, without differentiating between DCs and tumor cells. Cell viability decreased slightly during the next two days (data not shown). In several experiments, PEG-treated cells, as well as cells treated with high direct current, showed a slightly lower viability than cells treated with low direct current, a finding described by others for prostate and breast carcinoma cell lines (17).

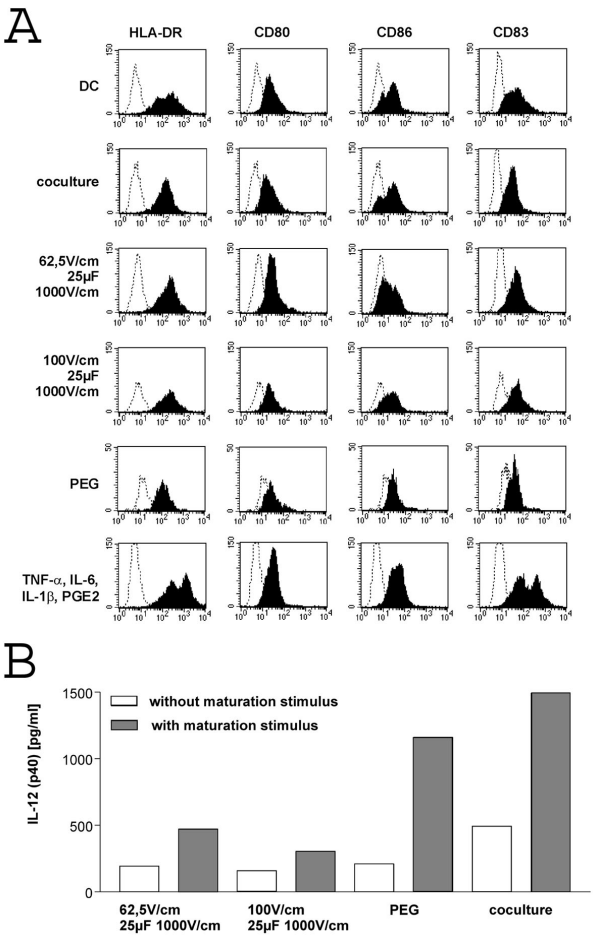

Figure 3. Antigen expression following fusion. (A) Immature DCs and melanoma (Mel Im) cells were subjected to electrofusion (using different parameters), as well as PEG fusion, and then put into culture. As controls, DCs were cultured alone or with the maturation-inducing cytokines TNF-alpha, IL-6, IL-1beta and PGE2. After 3 days, cells were harvested and stained with anti-HLA-DR-FITC, anti-CD83-PE, anti-CD86-FITC, anti-CD80-FITC or isotype-matched control mAbs. Additional staining was performed with anti-CD45-PerCP mAb to label DCs. Samples were analyzed by two-color flow cytometry gating for CD45-positive cells. To exclude staining artifacts, melanoma cells were added to the DC control sample for the staining procedure. (B) Dendritic and (Mel Im) melanoma cells were fused by electrofusion or PEG fusion and cultured in the absence and presence of TNF-alpha, IL-6, IL-1beta and PGE2 (to induce maturation). After 2 days, IL-12 (p40) secretion in the supernatant was determined by ELISA. Values are the mean of duplicates of one representative experiment (standard deviation<10%).

High expression of MHC and costimulatory molecules is essential for the induction of specific T cell responses. Expression in all PEG fusion and electrofusion settings determined four hours after treatment was comparable to that in untreated DCs (data not shown). It is known that simple manipulation of DCs can modulate surface antigen expression leading to a mature phenotype. However three days after fusion no upregulation of HLA-DR, CD80, CD83 or CD86 was detected, in contrast to cultures where maturation was induced by the cytokines TNF-alpha, IL-6, IL-1beta and prostaglandin E2 (PGE2) (18) (Figure 3A). DCs from some donors showed a rather high CD83 expression after mere pipetting as previously observed (19). Fewer cells seemed to express the antigens being evaluated in our samples treated with PEG or with a strong electrical pulse, which might be explained by the lower viability of these cells. Analysis of TNF-alpha or IL-12 (p40) secretion 24 and 72 h after fusion revealed no levels above background, in contrast to CD40Ligand-matured DCs which secreted both cytokines (data not shown). This again suggests that DCs are not driven into maturation by the fusion procedure itself.

Nevertheless, it was interesting to test whether DCs were still reacting to a typical maturation stimulus after the fusion procedure. A three-day incubation of cells having undergone the fusion procedure with TNF-alpha, IL-6, IL-1beta and PGE2 induced upregulation of HLA-DR and CD83 (data not shown), as well as secretion of IL-12 (p40) (Figure 3B). The lower secretion of IL-12 (p40) after cell fusion compared to that of coculture samples might again be attributed to a lower viability. IL-12 (p70) secretion was too low to be detected following the fusion conditions analyzed (data not shown).

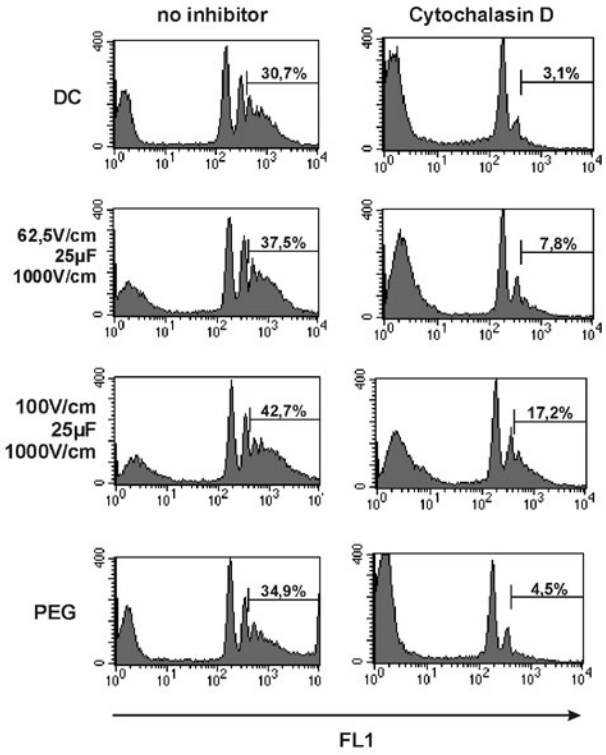

Figure 4. DCs are still able to engulf particles after the fusion procedure. DCs were treated with PEG or underwent electrofusion, while control cells were untreated. All samples were incubated with Fluoresbrite™ Carboxylated Microspheres (size 1.58 µm) for 150 min and analyzed by flow cytometry (left). Only those cells that had phagocytosed more than 2 beads were counted as positive (percentage indicated). As a control, a second set of samples was pretreated with 15 µM cytochalasin D prior to incubation with the microspheres (right). The fluorescence peak with an intensity of up to 10^1 represents DCs that have not internalized beads, as determined by a control sample (data not shown). One representative experiment is shown.

In addition to cytokine secretion we compared the phagocytic activity of DCs after different treatments. Figure 4 shows that in all settings 30-40% of the cells ingested more than 2 fluorescent particles. This could largely be blocked by cytochalasin D, a known inhibitor of phagocytosis (20). Colocalization of one or two beads was not very effectively blocked, probably due to unspecific binding of the beads. This might also be the reason for the inefficient blocking in samples treated with a strong pulse, which induced much cell aggregation. As already shown in Figure 1D, DCs in fusion samples not only took up small particles but were also able to ingest tumor cell material. Several groups have described the uptake of apoptotic tumor cells by DCs and its role in CTL induction (21, 22). The fact that we found tumor cell material ingested by DCs after fusion may therefore have a positive effect in vaccination approaches.

To assess the immunostimulatory capacity of cells after the fusion procedure, T cells were stimulated with autologous DCs fused with an allogeneic melanoma cell line. Cell yields of mononuclear cells after two weeks of culture revealed a 10-fold expansion after stimulation with fusion cells compared to an 8-fold expansion using coculture of dendritic and tumor cells. Stimulation with DCs or melanoma cells alone induced a 5-fold and 6-fold expansion respectively (data not shown). When the capability of DCs to stimulate specific T cells was directly compared either after fusion with allogeneic melanoma cells or by loading DCs with irradiated melanoma cells, both T cell lines displayed high levels of cytotoxicity against the melanoma cells (Figure 5A). Control settings using melanoma cells or DCs alone as stimulators induced a much lower T cell activity. Lysis of an irrelevant renal cell carcinoma was not detected (Figure 5A). These results were confirmed by the IFN-gamma ELISPOT assay carried out after two rounds of prestimulation (data not shown). Of note, T cell activation was also detected when restimulation was carried out during the assay with melanoma cells alone, while no response was observed with an irrelevant tumor (Figure 5B).

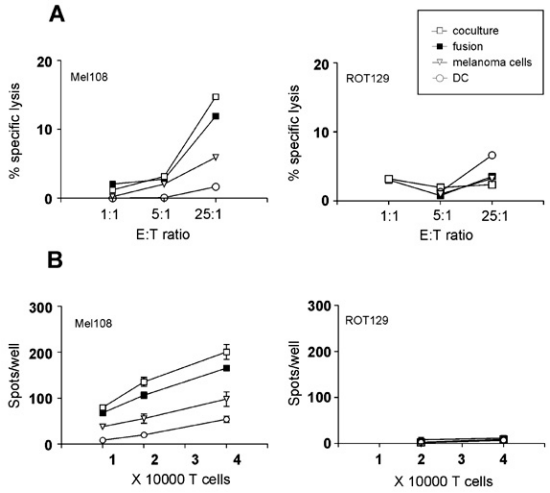

Figure 5. Activation of T cells after stimulation with DC-tumor cell hybrids. (A) T cells were stimulated with cells prepared from autologous DCs and allogeneic (Mel 108) melanoma cells. Electrofusion was performed at 50 V/cm combined with a 25 µF - 1000 V/cm pulse. Cocultures of DCs and tumor cells, as well as of DCs and melanoma cells (Mel 108) were used as control samples. After two weekly cycles of stimulation, T cells were tested for their cytotoxic activity in a 4-h ^{51}Cr-release assay against Mel 108 cells. Values represent triplicates at E:T ratios of 1:1, 5:1 and 25:1 (left). For specificity controls, T cells were tested for their cytotoxic activity against irrelevant ROT129 tumor cells (right). (B) The frequency of IFN-gamma secreting T cells in response to fused cells was determined in an ELISPOT assay. T cells were prestimulated for two weeks *in vitro* with either electrofused cells, a coculture of DCs and melanoma cells, DCs alone, or melanoma (Mel 108) cells alone as described in 5A. Restimulation during the assay was performed with Mel 108 cells. Results are shown as the mean number of spots per well (left). For specificity controls, restimulation was performed with irrelevant ROT129 tumor cells (right).

Discussion

Our PEG fusion results are consistent with the low fusion efficiency described by others (10, 23). Concerning electrofusion, Scott-Taylor *et al.* have shown comparable results with carcinoma cell lines derived from prostate and breast carcinoma fused to DCs cultured from CD34+ cells (17). Overall, we found a four- to eight-fold higher percentage of dual-fluorescent cells by flow cytometry after electrofusion compared to PEG treatment of cells previously stained with dye. Comparable fusion efficiencies were observed for fusions of tumor cells with immature and mature DCs (data not shown). We also tried to investigate the number of hybrid cells by flow cytometry of antibody-labeled cells. However, due to differences in the background fluorescence of dendritic and tumor cells, a clear distinction between stained and unstained cell populations after cell fusion was difficult (data not shown). Besides cell aggregates and DCs having phagocytosed tumor cell debris (5-10%), only up to 5% real hybrids could be detected by examination with a fluorescence microscope. Nevertheless, the absolute number of hybrid cells necessary for successful vaccination *in vivo* is not yet known and needs to be determined in additional studies, preferably in animal models.

In line with our experiments, it has been described that cocultures of tumor cells and DCs can also induce T cell activation (24). We have found that mere coculture of dendritic and tumor cells resulted in up to 5% dual-fluorescent cells. These cells (DCs having phagocytosed tumor cell debris and cell aggregates) can probably lead to some CTL activation by cross presentation. The advantage of hybrids, however, is the possible coexpression of MHC class I and class II molecules. This may lead to strong T cell help which has clearly been shown to be necessary for antitumor immune responses by CTLs (12, 25, 26). Because no clear superiority of a fusion vaccine compared to simple DC-tumor cell cocultures could be demonstrated in our *in vitro* assays, immunological effects of fusion vaccines should be investigated further in animal models.

Initial clinical trials using DC-tumor cell fusion have shown encouraging results (16, 27), however, independent confirmation by other groups is still pending. The cells in this study an unimpaired phenotype after the fusion procedure. As they were still able to induce a tumor-specific T-cell response *in vitro* they deserve further investigation as tools for immunotherapy.

Abbreviations

PEG, polyethylene glycol; PGE2, prostaglandin E2

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Materials and methods

Cell culture and preparation of monocyte-derived DCs

ROT129 is a renal cell carcinoma line obtained from a primary tumor, minced mechanically and subjected to digestion with an enzymatic cocktail as previously described (28). Melanoma cell lines Mel Im (29) and Mel 108 (30), as well as ROT129, were cultured in complete medium consisting of RPMI 1640, 0.22 mg/ml L-glutamine, 1 mmol/l sodium pyruvate, 50 mg/ml streptomycin, 50 U/ml penicillin (all from Gibco, Paisley, New Jersey) and 10% fetal calf serum (BioWhittacker, Belgium). Fresh tumor cells from a melanoma metastasis were isolated as described previously (28) and early passages were cryopreserved for later use.

Peripheral blood monocytes from healthy donors were isolated by leukapheresis, Ficoll-Hypaque and elutriation as described previously (31). DCs were generated by culturing monocytes in complete medium supplemented with 500 U/ml IL-4 (Schering-Plough, Kenilworth, New Jersey) and 500 U/ml GM-CSF (Novartis AG, Switzerland). DCs were harvested as nonadherent cells after 6-8 days (32). DC maturation was induced by further incubation for three days with TNF-alpha (10 ng/ml, Promocell, Heidelberg), IL-6 (1000 U/ml, Promocell),

IL-1beta (10 ng/ml, Promocell) and PGE2 (1 µg/ml, Pharmacia) (18). Elutriation fractions obtained at lower flow rates were used as a source of T lymphocytes (31).

Monoclonal antibodies and flow cytometry

Cells were immunostained using the following mAbs: anti-HLA-DR-FITC (B-F1, Dianova, Hamburg, Germany), anti-CD83-PE (HB15A, Immunotech, Marseille, France), anti-CD80-FITC (BB1, PharMingen, Hamburg, Germany), anti-CD86-FITC (2331 [FUN-1], PharMingen), anti-CD45-PerCP (Anti-Hle-1, Becton Dickinson), or isotype-matched control antibodies (Becton Dickinson, Mountain View, CA). After fixing the cells with 1% paraformaldehyde, surface marker analysis was performed using a FACScan (Becton Dickinson).

Fusion of dendritic and tumor cells

For polyethylene glycol-induced fusion, tumor cells and DCs were washed with PBS and $3x10^6$ tumor cells admixed to $3x10^6$ DCs. Cells were incubated for 2 min in 50% PEG (Merck, Darmstadt, Germany) in serum-free RPMI 1640 medium. After slow dilution of the PEG in the medium, cells were washed twice and resuspended in complete medium supplemented with IL-4 and GM-CSF. For electrofusion, tumor cells and DCs were washed twice with PBS, once with 5% glucose solution (Braun, Melsungen, Germany) and mixed in a fresh cuvette (Biorad, 0.4 cm gap). Fusion was performed by applying a direct current of 50, 62.5 or 100 V per cm for 5 seconds to induce cell alignment and a fusogenic high voltage pulse (25 µF - 1000 V/cm) driven by a Gene Pulser (Bio-Rad Laboratories, CA) (17). After fusion, cells were incubated in complete medium supplemented with IL-4 and GM-CSF.

Detection of vital hybrid cells by flow cytometry or fluorescence microscopy

To detect hybrid cells, tumor cells were stained either with 2 µM CMFDA (Molecular Probes, Leiden, The Netherlands) for flow cytometric analysis or with 200 µM CMFDA for fluorescence microscopic analysis. DCs were stained with 4 µM CMTMR (Molecular Probes) for both applications. A filter set for the fluorescence microscope was used to detect both wavelengths (Leica, Bensheim, Germany).

Preparation and analysis of cells by digital confocal fluorescence microscopy

To detect hybrid cells, cells were stained with anti-CD11c-FITC/anti-CD36-FITC mAbs (3.9/SMphi, Cymbus Biotechnologies, Hampshire, UK), as well as anti-Mel1 mAb (R24, Signet Pathology Systems, MA) using a Rhodamine Red-conjugated secondary antibody (Molecular Probes) after the fusion procedure. Nuclear counterstaining was performed with Hoechst 33342 (10.2 µg/ml; Sigma). To detect phagocytosis events, tumor cells were stained with PKH2-GL (20 µM, Sigma) prior to treatment. Following the fusion procedure, cells were stained with mAbs against CD1a (BL6, Coulter, Florida) and CD36 (FA6-152, Immunotech) using a Rhodamine Red-conjugated secondary antibody (Molecular Probes). Images were obtained with a microscope (Axiovert S100, Zeiss, Oberkochen, Germany) using 3 different filters (360±20 nm, 480±15 nm and 560±20 nm for excitation and 460±25 nm, 535±20 nm and 630±30 nm for emission). Each color was recorded separately and mathematically reassigned from blurring and autofluorescence, with the images superimposed at the end.

Determination of cytokine secretion

Cell samples from fusion experiments were cultured at a concentration of $1x10^6$ cells per ml in complete medium supplemented with IL-4 and GM-CSF as indicated above. Supernatants were harvested 24 and 72 h later, filtered (0.2 µm) and frozen. Cytokine secretion was determined using a commercially available ELISA set (PharMingen).

Determination of phagocytosis by FACScan analysis

PEG-treated or electro-pulsed immature DCs ($1x10^6$) were incubated for 150 min with $2x10^7$ Fluoresbrite™ Carboxylated Microspheres (1.58 µm, Polysciences Inc., Warrington, PA). Control samples were preincubated with 15 µM cytochalasin D (Sigma). Cells were washed twice with PBS and immediately analyzed by flow cytometry.

ELISPOT assay

Nitrocellulose plates (96-well, Millipore, Bredford, MA) were coated with anti-human IFN-gamma mAb (8 µg/ml, PharMingen) and blocking was performed with Basal Iscove Medium (Biochrom) supplemented with 10% AB serum. T cells stimulated *in vitro* for two weeks with either electrofused cells or control stimulator cells were incubated with stimulator cells for 20 hours at 37°C. Plates were then washed with 0.05% Tween 20 (Sigma) in PBS and incubated with biotinylated anti-human IFN-gamma mAb (0.3 µg/ml, PharMingen). After incubation with streptavidin-alkaline phosphatase (BioRad, Munich, Germany) and BCIP/NBT substrate (BioRad), plates were washed under running water and air-dried. Spots were counted using a stereo microscope (Zeiss).

Chromium release assay

T cell cytotoxic activity was measured in a conventional 4-h ^{51}Cr release assay. Melanoma (Mel 108) or renal cell carcinoma (ROT129) cells were used as target cells. E:T ratios were 25:1, 5:1 and 1:1, with 2000 target cells/well. Triplicate wells were averaged and the percent specific cytotoxicity was calculated as follows:

$$\% \text{ specific cytotoxicity} = [(\text{sample release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100]$$

For spontaneous release, targets were plated in the absence of T cells in complete medium supplemented with 10% human AB serum, instead of FCS. For maximum release, targets were plated in the presence of detergent (0.15% TritonX-100, Sigma).

Contact

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