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Fused Late Endocytic Compartments and Immunostimulatory Capacity of Dendritic–Tumor Cell Hybridomas

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Abstract Late endocytic compartments, containing MHC class II molecules in antigen presenting cells, fuse to each other in order to deliver antigens to these molecules. We have shown previously that fusion of late endocytic compartments takes place also in hybridomas. Therefore, we investigate here whether the level of fused late endocytic compartments affects the immunostimulatory capacity of hybridomas obtained by the electrofusion of dendritic and tumor cells. The level of fused late endocytic compartments in a single hybridoma cell was assessed and samples of electrofused cells were then cocultured with autologous T cells, resulting in the priming of naïve T cells. To test the immunostimulatory capacity of hybridoma cells, T-cell-induced cytotoxicity of tumor cells was assayed. The results demonstrate that in vitro cytotoxic T cell responses are enhanced if a higher percentage of fused late endocytic compartments is present in the cell population of electrofused hybridoma cells.

Keywords Cancer · Confocal microscopy · Hybridoma cells · Fusion of late endocytic compartments · Quantitative three-dimensional colocalization

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Dendritic cells (DCs) are the most effective antigen-presenting cells, which exhibit increased surface expression of major histocompatibility complex (MHC) class I and II antigens as well as costimulatory molecules required for efficient presentation of self and antigenic peptides, all required for the stimulation of T cells. In the past many strategies have been examined to introduce tumor antigens into and onto DCs. One of the most effective ones involves the fusion of DCs with whole tumor cells (TCs). Using this approach, DC-TC hybridomas are created, which are capable of expressing a wide spectrum of unaltered tumor antigens in the context with antigen-presenting molecules expressed by DCs. Hybridomas are therefore unique in their ability to stimulate a balanced CD4+ and CD8+ T cell response, which is important for effective and durable antitumor immunity (Rosenblatt et al. 2005).

The major advantage of DC-TC hybridomas over DCs loaded with tumor-derived antigens is that hybridomas contain known and unknown tumor antigens due to continuous translation of tumor-derived mRNA in the cytoplasm of fused cells (Galea-Lauri et al. 2002). Endogenously produced peptides are transported to the endoplasmic reticulum, where they are presented through the MHC class I pathway. Alternatively, tumor proteins can also be expressed by DC-derived-MHC class II heterodimers, which acquire their peptides from late endocytic vesicles. Although this could be explained partly by endolysosomal processing of tumor membrane proteins, the mechanisms for the generation of peptides from endogenous proteins for loading on MHC class II molecules are poorly understood (Mukherjee et al. 2001).

In our previous study we showed that spontaneous fusion of late endocytic compartments occurs in a hybridoma cell (Gabrijel et al. 2008). Therefore, the generation of tumor peptide–MHC class II complexes may result from

the fusion of late endocytic compartments containing tumor peptides derived from TCs and MHC class II-enriched compartments (MIICs) that originate from DCs. MIICs are the intracellular storage sites of MHC class II molecules in antigen-presenting cells, which have the characteristics of late endocytic multivesicular bodies or late endocytic compartments (Peters et al. 1991; Kleijmeer et al. 1997; Geuze 1998). In MIICs, MHC class II molecules are potentially loaded with peptides that are derived from late endocytic compartments via direct organelle fusion (Geuze 1998; Ferrari et al. 1997; Mellman et al. 1998). Previously, we have shown that by measuring the extent of fused late endocytic compartments, one can determine the level of fusion between cells (Gabrijel et al. 2008).

The present study tests the hypothesis that the extent of fused late endocytic compartments in a cell population of DC-TC hybridomas is associated with the level of in vitro cytotoxic T cell immune responses. To observe fusion events we labeled late endocytic compartments of DCs and human colorectal carcinoma Caco-2 cells with either red or green fluorescent dextran prior to their exposure to high-voltage electric pulses. Optimal electrofusion parameters were determined experimentally. The level of fused late endocytic compartments in a single hybridoma cell was determined by quantification of the degree of pixel colocalization in the confocal image (Kreft et al. 2004). We used a novel confocal microscopy-based approach to determine the correlation between the immunostimulatory capacity of hybridoma cells and the number of fused late endocytic compartments in a hybridoma cell population. The results presented here demonstrate that the in vitro cytotoxic T cell response is enhanced when a higher percentage of fused late endocytic compartments is present in the cell population.

Materials and Methods

Tumor Cell Cultures

Caco-2 (ATCC HTB 37), a human colorectal carcinoma cell line, was cultured in 25-cm^2 plastic tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) with 10% fetal calf serum (FBS; Sigma, St. Louis, MO), 1 mM L-glutamine (Gibco), 1 U/ml penicillin, and 1 μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Generation of Dendritic Cells

Human immature DCs were generated in vitro from peripheral blood-derived HLA-$A2^+$ monocytes of healthy volunteers as described previously (Bergant et al. 2006). After 5 days of culture, immature DCs were harvested and characterized by flow cytometry as $CD1a^{hi}$, $CD83^{low}$, $CD86^{low}$, and HLA-DR^{low}. Immature DCs were cultured for additional 20 h in the same medium that was used for DC generation, but this time supplemented with 20 ng/ml LPS (Sigma). The resulting mature DCs were then harvested and phenotypically characterized by flow cytometry as $CD86^{hi}$, $CD80^{hi}$, $CD83^{hi}$, HLA I^{hi}, and HLA-DR^{hi} cells.

Labeling Conditions for Late Endocytic Compartments

Cells were incubated with 0.5 mg/ml Alexa Fluor 546 or 0.5 mg/ml Alexa Fluor 488 dextrans (10,000 MW; Molecular Probes, Eugene, OR) for 13 h at 37°C. The dextran uptake was stopped by rinsing the cultures three times with the culture medium at 37°C. To clear marker from the early endosomal compartment, cultures were incubated for an additional 3 h at 37°C in marker-free medium.

Immunocytochemistry

We used anti-human HLA-DM monoclonal primary antibodies (BD Biosciences). For immunolabeling, the cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 15 min. Fixation was performed at room temperature. After four rinses in PBS, the nonspecific background staining was reduced with blocking buffer containing 3% bovine serum albumin (BSA) and 10% goat serum in PBS. The cells were then stained with primary and then secondary antibodies (conjugated with the fluorescent dye Alexa Fluor 488 [green; Molecular Probes]), both diluted into 3% BSA in PBS, and incubated at 37°C. Then they were mounted using a Light Antifade Kit (Molecular Probes).

Electrofusion

Stained cells were harvested by treatment with 4% trypsin. Equal proportions of green and red fluorescing cells were washed three times in electrofusion buffer (slightly conductive: 120 μS/cm ± 10%, at 23°C, 0.1 mM calcium acetate, 0.1 mM magnesium acetate, 1 mg/ml BSA, pH 7.2 ± 0.2; iso-osmolar, 285.0 ± 10 mosmol/kg; Isoosmolar Electrofusion Buffer; Eppendorf, Hamburg, Germany). We used an Eppendorf Multiporator, the helix fusion chamber (250 μl) consisting of a conically tapering core and platinum electrodes. We also used a custom-made planar fusion chamber, holding up to 5 ml of cell suspension, with a transparent bottom for observation of the ongoing fusion procedure under the microscope in real time. Close contact between cells was induced by

dielectrophoresis for 30 s in the field of 350 V/cm and 2 MHz. Subsequently fusion was performed by applying a high-voltage pulse (650 V/cm) for 30 μs and then the cells were exposed to a postalignment field of 350 V/cm and 2 MHz for 30 s. The optimal electrofusion parameters were determined experimentally. Samples were incubated in standard saline solution for 20 h at 37°C. The resulting cell suspension was examined for the presence of DC-Caco-2 hybridomas by confocal microscopy. The control cells were treated in the same way, except that exposure to the electric field was omitted.

Confocal Microscopy

z-stacks were acquired for each DC-Caco-2 hybridoma cell detected using a Zeiss LSM 510 confocal microscope with a Plan-Apochromat DIC objective (63×; NA = 1.4). Alexa Fluor 488 dextran was excited with an argon laser at 488 nm, and Alexa Fluor 546 dextran with a He/Ne laser at 543 nm. BP 505- to 530-nm and LP 560-nm emission filters were used to separate the red and green fluorescence. The sequential collection mode of image acquisition was used to prevent channel crosstalk.

Determination of the Number of Late Endocytic Compartments per Cell

The number of late endocytic compartments was counted as described previously (Gabrijel et al. 2008). Briefly, confocal microscopy was used to generate *z*-stacks of randomly selected nonelectrofused dextran-labeled cells. The average number of late endocytic compartments per cell was obtained by taking into account the average diameter of late endocytic compartments and the average thickness of optical slices. The diameters of 40 randomly selected late endocytic compartments were measured manually using LSM Carl Zeiss software. The step size (*z*-axes) of the optical slices was 0.5 μm, according to the Nyquist criterion. Late endocytic compartments were quantified using the ParticleCO program (Kreft and Zorec 2005) under the assumption that each compartment can appear in three adjacent optical slices.

Determination of the Yield of DC-Caco-2 Hybridoma Cells

The percentage of hybridomas was estimated by counting cells that contained at least a minimal number of red, green, or yellow late endocytic compartments and usually at least two nuclei. At least 300 randomly selected cells were considered in the analysis of a sample. The image of the cells was scanned systematically from the top left corner by an evaluation square (one-ninth of the area of the image) in which all the cells were counted starting from the row on the left, counting to the right, line after line. When the counting in the evaluation square was completed, the field was moved one step to the right and the procedure was repeated until the whole image was analyzed. The percentage of DC-Caco-2 hybridoma cells was expressed as the percentage of cells containing at least 30 red and 30 green or at least 30 yellow late endocytic compartments and usually at least two nuclei, in relation to all the cells counted.

Determination of the Extent of Fused Late Endocytic Compartments in DC-Caco-2 Hybridoma Cells

The average percentage of colocalized pixels in each optical slice was assessed by automated evaluation of colocalized pixels relative to all red and green fluorescent pixels (Kreft et al. 2004). The contrast of all images was uniformly set by linearly reassigning the value of the pixel intensities to use the full 8-bit range (0–255). We determined the threshold value, which separated the background intensity levels from the signal of single red and green pixels. The value 51 AU of 255 intensity levels, which corresponds to 20% of the maximum intensity level, was selected.

In Vitro Cell-Mediated Cytotoxicity Assay

The efficiency of DC-Caco-2 hybridomas to induce specific antitumor cytotoxic T cell responses was evaluated in a standard ^{51}Cr cytotoxicity assay. Lymphocyte fractions obtained during monocyte isolation from peripheral blood mononuclear cells were cocultured with hybridomas (autologous DCs-Caco-2 TCs; both HLA-A2^{+}) at a stimulator-to-effector ratio of 10:1 in RPMI 1640 (Cambrex) supplemented with 10% FBS and 20 U/ml rhIL-2 (Roche, Mannheim, Germany). After 7 days of culture, T cells were restimulated with the same DC-Caco-2 hybridomas and half of the medium was replaced with fresh medium. Seven days later, T cells were counted and used as effector cells in a standard ^{51}Cr cytotoxicity assay. The cytotoxicity was tested on Caco-2 cell line as a specific target, while autologous lymphoblasts were used as nonspecific targets. All target cells were labeled with $Na^{51}CrO_4$ (3 MBq per 1×10^6 cells; GE Healthcare, Czech Republic) and added to effector T cells at an effector-to-target (E:T) ratio of 5:1. The extent of specific cytotoxicity was determined as the amount of ^{51}Cr released from destroyed target cells during a 4-h coculture at 37°C in an air atmosphere enriched with 5% CO_2. In all experiments, the spontaneous ^{51}Cr release was bellow 20% of the total release. The percentage of specific cytotoxicity was calculated as follows: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Cell-mediated cytotoxicity

assays were performed in four independent experiments using three different donors of DCs and T cells.

Statistical Analysis

We used Student's *t*-test for statistical analysis of all treated and control samples. The α value was set at 0.05.

Results

Fluorescent Dextran Labels Late Endocytic Compartments in DCs and Caco-2 TCs

To assess the immunostimulatory capacity of DC-Caco-2 hybridomas, we labeled the same number of DCs and Caco-2 TCs with either 0.5 mg/ml red fluorescent Alexa Fluor 546 or 0.5 mg/ml green fluorescent Alexa Fluor 488 dextran (Deng et al. 1991). To confirm that dextran molecules are located in late endocytic compartments, DCs were first preloaded with red fluorescent dextrans (Fig. 1a). Subsequently they were stained with anti-HLA-DM monoclonal antibodies and the appropriate secondary antibodies, conjugated with the green fluorescent dye Alexa Fluor 488 (Fig. 1b). HLA-DM molecules are located in late endocytic compartments, where they facilitate binding of antigenic peptides on MHC class II molecules (Liljedahl et al. 1996; Perou and Kaplan 1993). Figure 1c represents the overlay of images in Fig. 1a and b and demonstrates that almost-equal amounts of red and green fluorescence colocalize in cytoplasmic organelles, which therefore appear yellow. The extent of the colocalized area of HLA-DM and dextran molecules was determined by estimating the level of colocalized red and green pixels, relative to all red fluorescent pixels detected in each optical slice of the acquired *z*-stack of individual randomly selected DC. The average percentage of colocalized pixels in the analyzed cells was 69% $\pm$ 6% ($n = 10$).

Generation of DC-Caco-2 Hybridomas

To obtain the optimal fusion rate, the electrofusion of red and green fluorescently labeled DCs and Caco-2 TCs, respectively, was performed by varying the electric field intensities (Fig. 2). The percentage of hybridoma cells in each sample was determined after overnight incubation at 37°C. The highest fusion rate of 9.4% $\pm$ 1.5% ($n = 416$) was achieved when the field intensity was set to 650 V/cm, which is significantly higher in comparison to the values detected in the control samples, where hybridomas also appeared, probably due to spontaneous fusion (2.8% $\pm$ 0.2%; $n = 179$; $P = 0.02$). A DC-Caco-2 hybridoma cell was determined as a cell that contains at least the minimal number of red, green, or yellow late endocytic compartments (Fig. 3), determined previously as 30 (Gabrijel et al. 2008).

Percentages of Fused Late Endocytic Compartments in DC-Caco-2 Hybridoma Cells

After the electrofusion process, we acquired *z*-stacks for each hybridoma cell detected and then we measured the extent of fused late endocytic compartments in a single DC-Caco-2 hybridoma cell. Late endocytic compartments were considered fused if a double-labeled red and green compartment, appearing yellow, was detected among the red and green ones. To confirm that the yellow compartments were not due to overlay (overlapping) of red and green late endocytic compartments, *z*-stacks were recorded and images carefully analyzed by tracing the object extension into the *z*-axis. The estimated probability of false detection is extremely low (1×10^{-5}) (Gabrijel et al. 2008). The extent of apparently fused late endocytic compartments, containing red and green fluorescent dyes, was determined by calculating the number of red, green, and colocalized pixels for each optical slice in the acquired *z*-stack (Kreft et al. 2004). Colocalized pixels were then expressed as a percentage of all red and green pixels in each hybridoma cell. The percentages of colocalized pixels determined for each hybridoma cell were then averaged to obtain the general percentage of fused late endocytic compartments in DC-Caco-2 hybridoma cells incubated for 20 h at 37°C. The average percentage of fused late endocytic compartments was not statistically different in hybridomas detected in electrofused samples (37.3% $\pm$ 0.4%; $n = 37$), compared to the average value detected in control samples (36.1% $\pm$ 0.1%; $n = 5$). However, the absolute number of fused late endocytic compartments in the whole population of cells in the electrofused sample was significantly higher in comparison to the controls, where the number of DC-Caco-2 hybridomas was significantly lower.

The Number of Fused Late Endocytic Compartments in a Cell Population Indicates the Level of In Vitro Cytotoxic T Cell Responses

Finally, we analyzed whether the number of fused late endocytic compartments in a hybridoma cell population affects its immunostimulatory capacity after electrofusion. The influence of fusion rate of late endocytic compartments in electrofused samples on the specific antitumor cytotoxic T cell response was evaluated in a standard ^{51}Cr release cytotoxicity assay (Trevor et al. 2004). Autologous T cells, which were obtained from the same donor as DCs, were cocultured with electrofused DC-Caco-2 hybridomas. After restimulation with the same hybridomas, T-cell-mediated

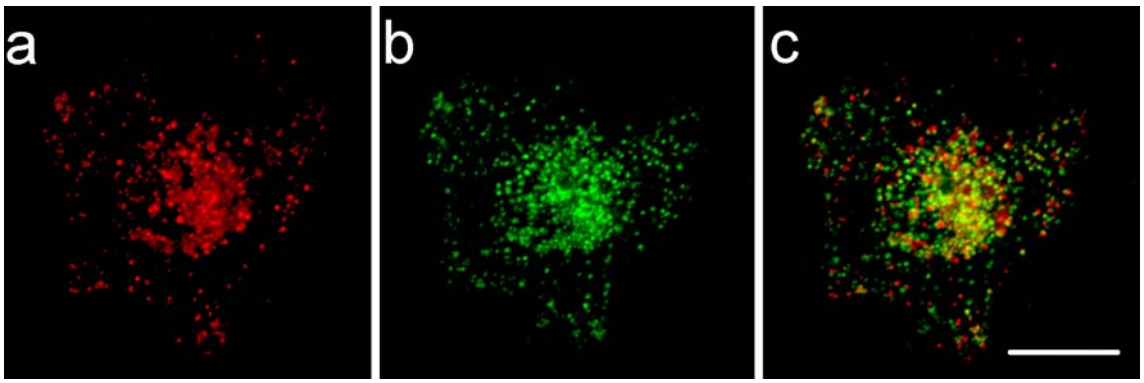

Fig. 1 Fluorescent dextrans are located in late endocytic compartments that are involved in antigen presentation pathway of dendritic cells (DCs). **a** Fluorescence image of a DC with late endocytic compartments loaded with Alexa Fluor 546 dextran for 13 h and chased for an additional 3 h in a dextran-free culture medium. **b** To demonstrate that dextran molecules are actually located in late endocytic compartments, involved in the antigen-presenting pathway, DCs were first labeled with anti-HLA-DM monoclonal antibodies and then with the secondary antibodies coupled with the green fluorescent dye Alexa Fluor 488. **c** Areas of colocalization appear in yellow in the composite image. The percentage colocalized pixels relative to all red pixels in this confocal image is 75%. Scale bar: 10 μm

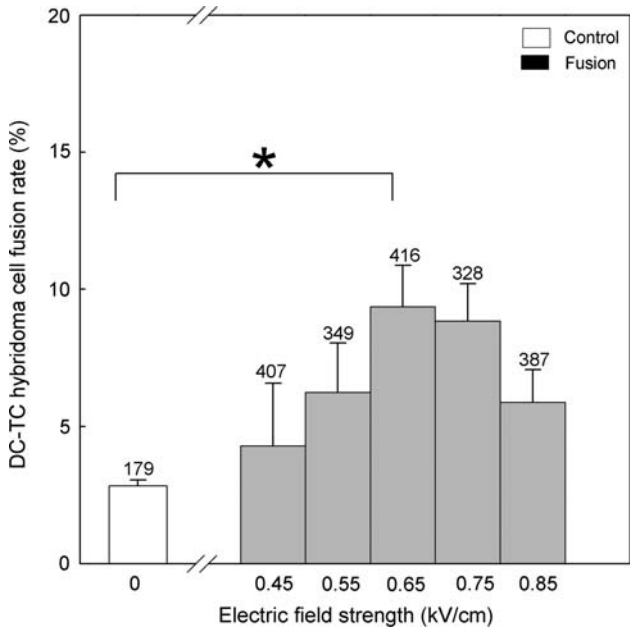

Fig. 2 DC-Caco-2 hybridoma cell fusion rates as a function of electric field intensity. Fluorescent dextran-loaded Caco-2 and DCs were pulsed once for 30 μs with electric fields of increasing strengths. Hybridoma cell fusion rates were plotted after 20 h of incubation at 37°C as a function of the electric field strength. Note that the optimal fusion rate was achieved when the field intensity was 650 V/cm. The level of hybridoma cell fusion rates detected in control samples, where hybridoma cells appear only due to spontaneous fusion, is statistically lower ($P = 0.02$) after incubation (20 h, 37°C)

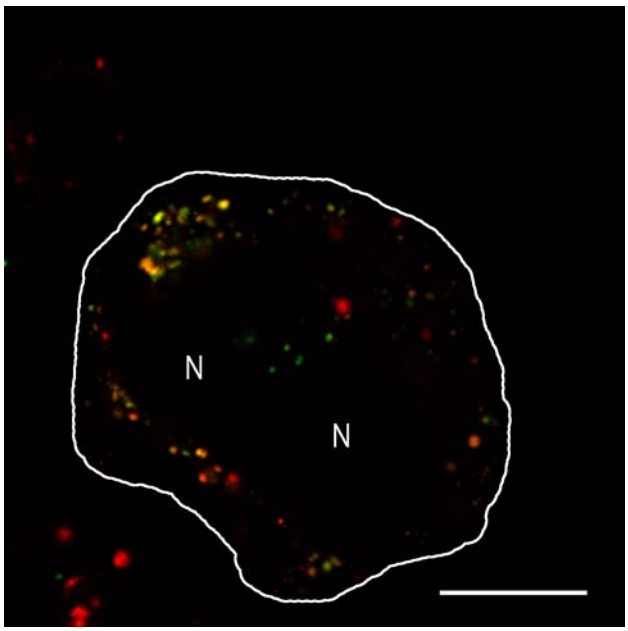

Fig. 3 Confocal microscope micrograph of a hybridoma cell generated by electrofusion. Caco-2, a human colorectal carcinoma cell line, was labeled with green fluorescent dextran and was fused with autologous DCs stained with red fluorescent dextrans. After overnight culture (37°C, 20 h) fused samples were analyzed for the presence of DC-Caco-2 hybridomas. The DC-Caco-2 hybridoma cell shown in this micrograph (*traced white*) contains 9 red, 7 green, and 48 late endocytic compartments stained with both dyes (which appear yellow or orange). The hybridoma cell has two nuclei (N). Scale bar: 15 μm

cytotoxicity was determined by measuring the amounts of released ^{51}Cr following the exposure of ^{51}Cr-labeled Caco-2 TCs as targets to effector T cells present in the stimulated cultures. Representative results of one of four independent cell-mediated cytotoxicity assays are displayed in Fig. 4a. The experimental evidence demonstrates that electrofused samples generate statistically stronger T cell responses (48.8% ± 1.2%; fus1; gray column) in comparison to controls that contain a mixture (mix, gray column) of unfused DCs and Caco-2 TCs (21.5% ± 0.6%; $P = 0.003$). Coculture of stimulated T cells with only Caco-2 TCs (15.6% ± 0.8%; TC; gray column) or with

DCs (9.9% ± 1.5%; DC; gray column) failed to generate any significant specific lysis. The data presented in Fig. 4a also show the lack of cytotoxic T cell activity against autologous lymphoblasts that were used as nonspecific targets (white columns) in each of the four independent experiments. In Fig. 4b the results of all seven experiments carried out with cells isolated from three different donors are shown. They unequivocally show that all electrofused samples containing DC-Caco-2 hybridoma cells stimulate statistically more efficient specific cytotoxic T cell responses in comparison to the mixture of unfused DC and Caco-2 cells. Finally, we also analyzed whether the number of fused late endocytic compartments in fused cell populations indicate their immunostimulatory capacity (induction of specific cytotoxicity). Therefore, we determined the percentage of fused late endocytic compartments in the whole cell population by multiplying the average fraction of fused late endocytic compartments in a single hybridoma cell by the fraction of all hybridoma cells in the cell population. Table 1 shows that the average percentage of specific lysis determined in controls (37.53% ± 5.43%; $n = 15$) increased significantly in electrofused samples, by approximately 60%, to 59.52% ± 6.04% ($n = 13$; $P = 0.011$), when the number of fused late endocytic compartments increased by approximately fivefold (from 0.80 ± 0.07%, $n = 179$ to 3.95 ± 0.64%, $n = 416$).

Discussion

In the present study, we investigated whether the level of fused late endocytic compartments in a cell population affects the potency of the in vitro cytotoxic T cell response, since DC-TC cell vaccines are thought to efficiently evoke increased responsiveness of T cells (Trevor et al. 2004). To evaluate specific cytotoxic T cell responses, DCs and human colorectal carcinoma Caco-2 cells were preloaded with red and green fluorescent dextrans, respectively, and then electrofused. Fluoresceinated, high molecular weight dextran accumulates in the phagosomes over a period of 24 h (Wang and Goren 1987). Antigen-presenting cells exhibit two distinct strategies upon antigen encounter. Macrophages contain high levels of lysosomal proteases and rapidly degrade internalized proteins; DCs, on the other hand, are protease-poor, which results in a limited capacity for lysosomal degradation (Delamarre et al. 2005). DCs in vivo degrade internalized antigens slowly, which favors antigen presentation. Delamarre et al. administered soluble protein antigens to mice. They coinjected fluorescent dextran as a nondegradable probe to identify all endocytic antigen-presenting cells. They found that after 20 h one-third of the dendritic cells still contained both the proteins and the dextrans, which favors antigen presentation.

Confocal analysis of *z*-stacks revealed that red fluorescent dextran molecules phagocytosed by DCs colocalize to a high degree with HLA-DM molecules (Fig. 1c). These molecules are localized in MIIC compartments and form complexes with MHC class II molecules. These results confirm that late endocytic compartments are involved in the antigen presenting activity of DCs (Cresswell 1996). Electrofusion of DCs and Caco-2 TCs was performed using an optimal electric field intensity, which was experimentally defined (Fig. 2). Following the electrofusion procedure and incubation stage, each sample was then added as a stimulator cell fraction to autologous T cells as responders. Following the restimulation step, the specific cytotoxicity of effector $CD8^+$ T cells was assessed by evaluation of the lysis of Caco-2 target cells (Trevor et al. 2004). In all experiments we found that the mixtures of unfused DCs and Caco-2 TCs were less effective than their DC-Caco-2 hybridoma counterparts in inducing specific cytotoxic T cell responses in vitro (Fig. 4a, b). Similarly, the specific cytotoxic T cell lysis of Caco-2 target cells following stimulation with solely DCs or Caco-2 cells was always found to be at the basal level, indicating that DCs have to process and present Caco-2 antigens within the context of their MHC class I and II molecules in order to efficiently stimulate specific cytotoxic T cell responses. The results also show that cytotoxic T cell activity against autologous lymphoblasts was negligibly low, indicating that there was no major response directed toward self-antigens, expressed on the surface of DCs.

Furthermore, the analysis showed that the average percentage of DC-Caco-2 hybridoma cells was statistically higher in electrofused samples versus the unfused population of DCs and Caco-2 TCs (controls). Interestingly, a low level of apparently spontaneous cell fusions was observed in the controls, which is consistent with the active role of DCs in the antigen presentation process (Strome et al. 2002). The confocal analysis of hybridoma cell *z*-stacks showed that fusion rates of late endocytic compartments were not significantly different in DC-Caco-2 hybridomas detected in electrofused versus unfused control samples. However, the absolute number of fused late endocytic compartments in electrofused samples was higher, due to a higher level of hybridomas detected in each experiment. Therefore, we examined whether the level of fused late endocytic compartments in a sample indicates the specific in vitro cytotoxic T cell response. The results demonstrate that there is a significant increase, by 60%, in the specific in vitro cytotoxic T cell response when the number of fused late endocytic compartments in a cell population increases (Fig. 4). These results therefore confirm the importance of postfusion incubation time for the preparation of DC-TC

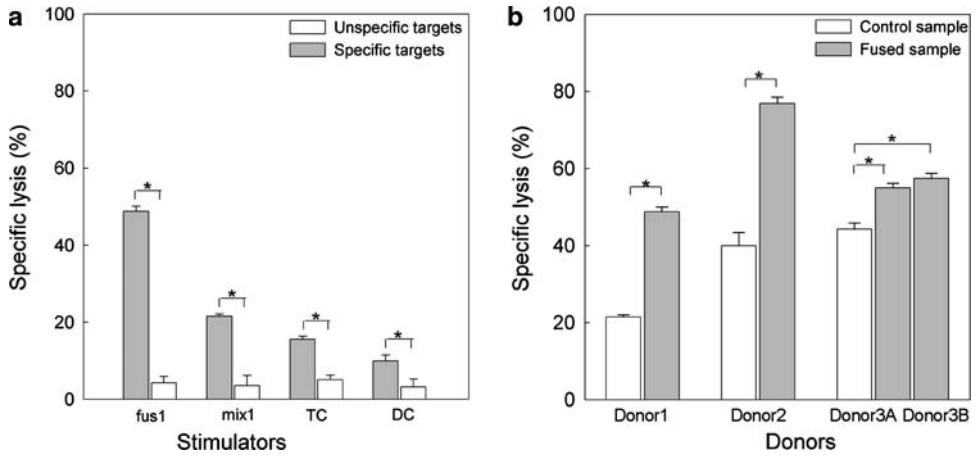

Fig. 4 Induction of in vitro T-cell-mediated cytotoxicity. **a** Hybridomas prepared by electrofusion of samples of dendritic cells (DCs) and Caco-2 TCs were used as stimulators of autologous (related to DC) T cells. The cytolytic activity of T cells was determined after the second stimulation in a standard ^{51}Cr-release assay, using tumor Caco-2 cells as specific targets and autologous lymphoblasts as nonspecific targets. The percentage specific cytotoxicity was calculated as follows: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. The cell-mediated cytotoxicity assay was performed in four independent experiments. Each value is expressed as the mean ± SE of at least three parallel cell cultures. The specific target cell (Caco-2 tumor cell [TC]) lysis experiment shown in the graph represents selected data from one of the four independent trials; DC-Caco-2 hybridomas (fus1; gray column) induce a more potent cytotoxic T cell response (% specific lysis) than the mixture of unfused DCs and Caco-2 TCs (mix1; gray column) as shown by the significantly higher percentage specific lysis of ^{51}Cr-loaded Caco-2 target cells. The cytotoxic T cell response was Caco-2 specific and not directed toward self-antigens as shown by the very low lysis of autologous lymphoblasts (white columns). Unfused DCs or TCs alone also failed to trigger cytotoxic T cell responses (DC, TC; gray columns). **b** Percentage specific lysis of Caco-2 target cells detected in seven separate experiments using cells from three different donors. DC-Caco-2 hybridoma cells (gray columns) in each experiment stimulate statistically more potent cytotoxic T cell responses in comparison to control samples that contained a mixture of unfused DC and Caco-2 cells as stimulators ($P < 0.01$)

Table 1 Comparison of the relative amount of fused late endocytic compartments in the cell population and the relative specific lysis in electrofused samples

	Fused late endocytic compartments in cell population (%)	Specific lysis (%)
Control	0.80 ± 0.07 ($n = 179$)	37.53 ± 5.43 ($n = 15$)
Electrofused	3.95 ± 0.64* ($n = 416$)	59.52 ± 6.04* ($n = 13$)
Fold change	5.10 ± 1.10	1.69 ± 0.26

Note: n, number of cells counted and number of samples, respectively The average percentage specific lysis determined in controls (37.53% ± 5.43%; $n = 15$) increased significantly in electrofused samples, by approximately 60%, to 59.52% ± 6.04% ($n = 13$; $P = 0.011$) when the number of fused late endocytic compartments increased from 0.80% ± 0.07% ($n = 179$) to 3.95% ± 0.64% ($n = 416$), * $P < 0.01$

hybridoma cell vaccine, which is required for the development of optimal dynamics of the antigen presentation process (Kleijmeer et al. 1995).

In conclusion, the data presented here show that the increased number of fused late endocytic compartments in a cell population increases the specific in vitro cytotoxicity of T cells. Thus, we considered that DC-TC hybridomas with fully fused late endocytic compartments are able to process and present tumor antigens more effectively than those incubated for shorter times after electrofusion. The results also indicate that the effectiveness of cytotoxic T cell immune responses after vaccination with DC-TC hybridomas correlates with the time of postfusion incubation of hybridomas at 37°C. Our study contributes to the efforts to improve the clinical efficacy of DC-TC hybridoma cellular vaccines.

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