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Tumor Cell Lysate-pulsed Human Dendritic Cells Induce a T-Cell Response against Pancreatic Carcinoma Cells: an *in Vitro* Model for the Assessment of Tumor Vaccines

Max Schnurr,[1,2] Peter Galambos,[1] Christoph Scholz, Florian Then, Marc Dauer, Stefan Endres, and Andreas Eigler[3]

Division of Clinical Pharmacology, Department of Medicine, Ludwig-Maximilians-University of Munich, 80336 Munich, Germany

ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells and play a pivotal role in T cell-mediated immunity. DCs have been shown to induce strong antitumor immune responses *in vitro* and *in vivo*, and their efficacy is being investigated in clinical trials. Compared with vaccination strategies directed against a single tumor antigen, tumor-cell lysate as the source of antigen offers the potential advantage of inducing a broad T-cell response against multiple known, as well as unknown, tumor-associated antigens expressed by the individual tumor. We used pancreatic carcinoma cell lines to develop an *in vitro* model for monitoring T-cell responses induced by lysate-pulsed DCs. Monocyte-derived DCs of HLA-A2$^+$ donors were pulsed with lysate generated from the HLA-A2$^+$ pancreatic carcinoma cell line Panc-1. In some experiments, the immunogenic protein keyhole limpet hemocyanin (KLH) was added to the lysate. Subsequently, the antigen-loaded DCs were activated with tumor necrosis factor-α and prostaglandin E_2. Autologous mononuclear cells were cocultured with DCs in the presence of low-dose interleukin (IL)-2 and IL-7 and were restimulated weekly with new DCs. High levels of IL-12 and IFN-γ could be detected in the supernatants, indicating a T-helper type 1-type immune response. This cytokine profile was associated with the expression of the activation marker CD69 on both T helper and CTLs and with an antigen-induced proliferative T-cell response. After 4 weeks, CTL-mediated cytotoxicity was assessed. Tumor cell lysis was specific for Panc-1 tumor cells and was MHC class I-restricted. Cytokine secretion, CD69 expression of T cells, and antigen-induced T-cell proliferation correlated with the cytotoxic activity and were more pronounced when KLH was added to the lysate. This is the first study to show that T cells specific for pancreatic carcinoma cells can be generated *in vitro* by lysate-pulsed DCs and that the T-cell response can be enhanced by KLH. This *in vitro* model can be applied to compare different strategies in the development of DC-based tumor vaccines.

INTRODUCTION

Current strategies in the treatment of metastasized pancreatic carcinoma offer little hope for a cure and have only slight impact on survival (1). Therefore, new treatment modalities are warranted for this type of cancer. Efforts in the immunotherapy of malignant disease concentrate on the induction and enhancement of immune responses against tumors. A promising approach is the use of DCs,[4] which are highly effective antigen-presenting cells with the unique capability of inducing primary immune responses against tumor-associated antigens (2). Animal studies (3, 4) and human cancer trials (5–9) have shown that specific T-cell responses against tumors as well as tumor regression can be achieved with vaccines based on DCs.

Potential targets for the immunotherapy of pancreatic carcinoma are antigens such as carcinoembryonic antigen (10, 11), HER-2/neu (12, 13), MUC-1 (14–17), mutant ras (18, 19), p53 (20, 21), and gangliosides (22). However, vaccinating against a single antigen has disadvantages, because it is unknown which of the identified antigens have the potential to induce an effective antitumor immune response. Furthermore, immunity against a single antigen may be ineffective in tumors with heterogeneous cell populations and carries the risk of inducing tumor antigen escape variants (7). In addition, this strategy is restricted to those patients with a specific HLA type.

The use of unfractionated tumor-derived antigens in the form of tumor cell lysates or whole tumor cells circumvents these disadvantages. Tumor lysates contain multiple known as well as unknown antigens that can be presented to T cells by both MHC class I- and class II-pathways (4, 23, 24). Therefore, lysate-loaded DCs are more likely to induce a polyclonal expansion of T cells, including MHC class II-restricted T-helper cells. These have been recognized to play an important role in the activation of CTLs, probably the most important cells in effecting an antitumor immune response (25). The generation of CTL clones with multiple specificities may be an advantage in heterogeneous tumors and could also reduce the risk of tumor escape variants. Furthermore, lysate from the autologous tumor can be used independently of the HLA type of the patient. A major drawback of unfractionated tumor antigens is the possibility of inducing an autoimmune reactivity to epitopes that are shared by normal tissues (26). However, in clinical trials using lysate or whole tumor cells as the source of antigen, no clinically relevant autoimmune responses were detected (6, 8, 27).

For the development of an effective tumor vaccine based on DCs, the influence of experimental parameters on the immune response remain to be determined, such as the optimal source and subtype of DCs, the choice of the tumor antigen preparation, methods for effectively introducing antigens into MHC class I- and II-processing pathways, and the use of activators and adjuvants. Because not all of these parameters can be optimized in controlled clinical trials, there is a need for *in vitro* models to address these questions.

In this study, we present an *in vitro* model designed in our laboratory to test different strategies for the development of a therapeutic vaccine against pancreatic carcinoma based on tumor lysate-pulsed DCs. Several parameters such as the cytokine profile, tumor antigen-induced T-cell proliferation, expression of T-cell activation markers, and cytolytic T-cell activity were assessed to characterize the immune response.

MATERIALS AND METHODS

Reagents. Recombinant human cytokine GM-CSF was purchased from Novartis (Basel, Switzerland), IL-4 from Promega (Madison, WI), IL-2 and IL-7 from Strathman Biotech (Hannover, Germany), and TNF-α from R&D Systems (Wiesbaden, Germany). PGE_2, KLH, and FITC-dextran were obtained from Sigma Chemical Co. (Munich, Germany). [^{3}H]thymidine was purchased from Amersham Buchler (Freiburg, Germany) and [^{51}Cr]sodium chromate from NEN Life Sciences (Zavantem, Belgium).

Cell Culture. Cell cultures from human peripheral blood mononuclear cells were maintained in RPMI 1640 culture medium (Biochrom, Berlin,

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[3] To whom requests for reprints should be addressed, at Medizinische Klinik Innenstadt, Ziemssenstrasse 1, 80336 Munich, Germany. Phone: 49-89-51602378; Fax: 49-89-51604406; E-mail: Andreas.Eigler@med.uni-muenchen.de.

[4] The abbreviations used are: DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor; PGE_2, prostaglandin E_2; KLH, keyhole limpet hemocyanin; MNC, mononuclear cells; NK, natural killer; Th-1, T-helper type 1.

Germany) supplemented with 2% human AB serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine (Life Technologies, Inc.), 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma Chemical Co., Munich, Germany). The human pancreatic carcinoma cell lines AsPc-1 (HLA-A2$^-$), Panc-1 (HLA-A2$^+$), and Capan-1 (HLA-A2$^+$) were purchased from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). The gastric carcinoma cell line KATO-III (HLA-A2$^-$) was obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 supplemented with 10% FCS (Biochrom), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Flow Cytometry Analysis. Antibodies used to phenotype the cells were anti-HLA-DR-PE, anti-CD80-PE, anti-CD86-PE, anti-CD83-FITC, anti-CD54-PE, anti-HLA-ABC-FITC, anti-CD14-FITC, anti-CD8-PE, anti-CD4-PE, anti-CD69-FITC (PharMingen, San Diego, CA), and anti-CD3-PerCP (Becton Dickinson, San Jose, CA). For staining, 10^5 cells were suspended in 100 μl of PBS and were incubated with 10 μl of the antibodies for 20 min on ice. Flow cytometric analysis was performed on a FACS-Calibur (Becton Dickinson).

Preparation of Tumor Cell Lysate. Confluent cultures of pancreatic carcinoma cell lines were incubated with 0.01% EDTA-solution for 10 min, carefully detached with a cell scraper, washed twice in PBS, and resuspended at a density of 5×10^6/ml in serum-free medium. The cell suspensions were frozen at −80°C and disrupted by four freeze-thaw cycles. For the removal of crude debris, the lysate was centrifuged for 10 min at $300 \times g$. The supernatant was collected and passed through a 0.2-μm filter. The protein concentration of the lysate was determined by a commercial assay (Bio-Rad, Munich, Germany). Lysates were tested for endotoxin contamination with the *Limulus* amoebocyte lysate assay (BioWhittaker) according to the manufacturer's instructions and were found to be negative (endotoxin content <1 pg/ml).

Generation of Tumor Lysate-pulsed Mature DCs. Monocyte-derived DCs were generated as described elsewhere (28). In brief, MNC from healthy HLA-A2$^+$ donors were isolated from peripheral blood by Ficoll-Hypaque gradient centrifugation and were subsequently allowed to adhere in culture flasks for 1 h. The initially adherent cell fraction was harvested and cultured in the presence of GM-CSF (1000 units/ml) and IL-4 (1000 units/ml). On day 6, the cells were incubated with lysate of pancreatic carcinoma cell lines at a final concentration of 120 μg of protein/ml for 3 h. In some of the experiments, KLH was added at a concentration of 25 μg of protein/ml. Subsequently, DCs were activated with TNF-α (1000 units/ml) and PGE_2 (1 μM) for 24 h.

Endocytic Activity. Endocytic activity of DCs was assessed by adding FITC-dextran (0.5 mg/ml) to the culture medium for 30 min at 37°C (control on ice). Thereafter, cells were extensively washed, and the uptake of FITC-dextran was analyzed by flow cytometry.

Coculture of DCs and T cells. Nonadherent MNC from HLA-A2$^+$ donors were cocultured with autologous DCs in three parallel groups consisting of unpulsed, lysate-pulsed, or lysate- plus KLH-pulsed DCs at a ratio of 20:1. Every 7 days the cells were restimulated with DCs, and low doses of IL-2 (10 units/ml) and IL-7 (5 ng/ml) were added to the culture medium. Fifty percent of the medium was replaced on days 3 and 5 after each stimulation by fresh medium containing 25 units/ml IL-2 and 10 ng/ml IL-7.

Cytokine Assays. Forty-eight h after restimulation with DCs, the supernatants of the cocultures were collected, and the concentrations of IL-12 (p40 and p70), IFN-γ, and IL-4 were measured by ELISA (Endogen, Woburn, MA).

Proliferative T-Cell Response. To test DCs for their capacity to induce a proliferation of allogeneic T cells, DCs were harvested and cocultured with 2×10^5 allogeneic nonadherent MNC/well in 96-well round-bottomed microtiter plates at ratios ranging from 1:20 to 1:320. To assess antigen-induced autologous T-cell proliferation, nonadherent MNC were removed from the cocultures after two stimulations with lysate- or lysate- plus KLH-pulsed DCs and restimulated with autologous unpulsed or lysate-pulsed DCs. On day 4, [^{3}H]thymidine (1 μCi/well) was added, and cells were harvested after 18 h. The amount of incorporated [^{3}H]thymidine was analyzed in a liquid scintillation counter (Wallac, Turku, Finland).

Cytotoxicity Assay. After four stimulations with DCs, the lytic activity of MNC was assessed in an 18-h ^{51}Cr-release assay. A suspension of single carcinoma cells was incubated with 100 μCi [^{51}Cr]sodium chromate/10^6 cells for 1 h and washed five times. Tumor cells (5×10^3)/well were incubated with nonadherent MNC from the cocultures at E:T ratios ranging from 80:1 to 10:1 in round-bottomed 96-well microtiter plates. After 18 h, 100 μl of supernatant of each well was collected, and radioactivity was measured with a gamma counter (Wallac Oy, Turku, Finland). Specific lysis was calculated by the formula: specific ^{51}Cr-release = [(experimental counts − spontaneous counts)/(maximal counts − spontaneous counts) × 100%]. To determine MHC class I-restriction of tumor cell lysis in some assays, the target cells were preincubated with the MHC class I-blocking antibody W6/32 (Serotec, Oxford, United Kingdom).

RESULTS

Testing of the Tumor Cell Lysates. Generation of monocyte-derived DCs was highly reproducible. After a single adhesion step and 5 days of culture in the presence of GM-CSF and IL-4, the yield of DCs was approximately 8–15% of the isolated peripheral blood mononuclear cells. DCs presented as clusters of large cells with stellate morphology. DCs strongly incorporated FITC-dextran, expressed CD54, MHC class II, and low levels of CD80 and CD86 (Fig. 1). Upon a 48-h activation period with TNF-α and PGE_2, DCs were characterized by a low endocytotic activity for dextran, high expression of costimulatory molecules, MHC class II and CD83 (Fig. 1), secretion of IL-12 (Fig. 3), and an enhanced T-cell stimulatory capacity (Fig. 2; *unpulsed*).

On day 5, DCs were pulsed with lysate from the three pancreatic carcinoma cell lines AsPC-1, Panc-1, and Capan-1 at protein concentrations ranging from 30–480 μg/ml. Viability of lysate-pulsed DCs was assessed by propidium iodide-staining and the exclusion of trypan blue dye. Lysate concentrations of 240 μg/ml or more were found to be toxic, whereas 120 μg/ml (equivalent to 1.3 tumor cells/DC) or less did not influence viability (data not shown).

Next, we assessed the T-cell stimulatory capacity of lysate-pulsed DCs in an allogeneic mixed lymphocyte reaction. Interestingly, the pulsing of DCs with lysate of three pancreatic carcinoma cell lines had different effects on DC function (Fig. 2). Lysate of AsPc-1 activated

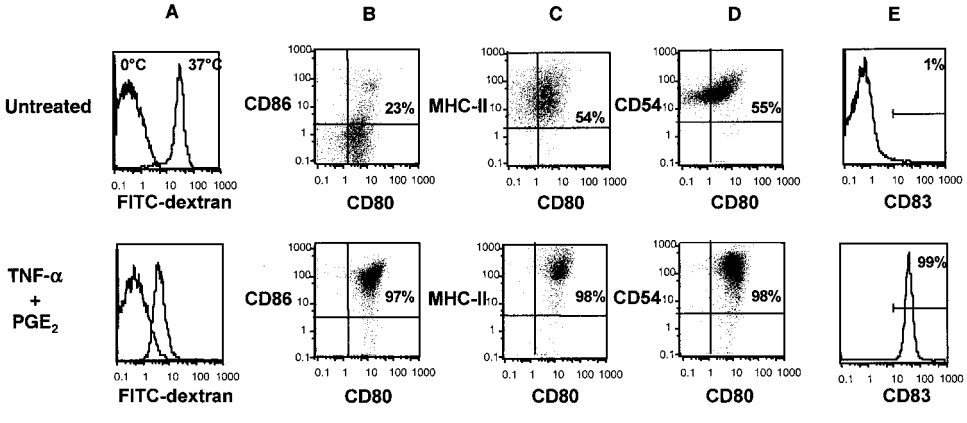

Fig. 1. Decrease of endocytotic activity and changes in phenotype of DCs by stimulation with TNF-α and PGE_2. On day 5 of culture, DCs are highly endocytotic (*A*, uptake of FITC-labeled dextran) and express low levels of CD80 and CD86 (*B*) and intermediate levels of MHC class II (*C*) and CD54 (*D*). After a 48-h incubation in the presence of TNF-α and PGE_2, DCs lose their ability to incorporate dextran, up-regulate CD80, CD86, CD54, and MHC class II, and express the maturation marker CD83 (*E*).

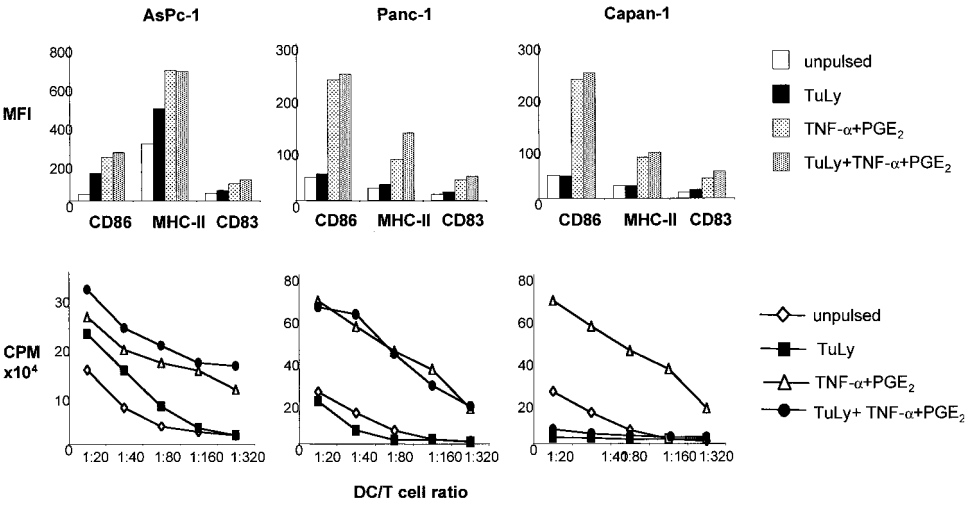

Fig. 2. Pancreatic carcinoma cell lysates from cell lines differentially influence DC surface marker expression and T-cell-stimulatory capacity. Day-5 DCs were pulsed with lysate from one of three pancreatic carcinoma cell lines (AsPc-1, Panc-1, or Capan-1) at a concentration of 120 μg of protein/ml for 3 h and were subsequently activated with TNF-α and PGE_2 or left untreated. Surface marker expression was assessed by flow cytometry and T-cell-stimulatory capacity in an allogeneic mixed lymphocyte reaction. Representative data of three independent experiments are shown.

DCs, which was reflected by an increased expression of costimulatory molecules and MHC-II as well as an enhanced T-cell proliferation rate. However, CD83 expression was not induced. In contrast, lysate of Capan-1 completely inhibited the T-cell stimulatory capacity of DCs, without altering the surface marker expression. Subsequent activation of DCs with TNF-α and PGE_2 induced maturation, but the DCs were still dysfunctional. Pulsing DCs with lysate of Panc-1 did not significantly influence surface marker expression or T-cell proliferation rates, and an activation with TNF-α and PGE_2 markedly improved their T-cell stimulatory capacity.

For the long-term cocultures of DCs with T cells, we used lysate of the HLA-$A2^+$ cell line Panc-1 to load immature DCs of HLA-$A2^+$ donors at a final concentration of 120 μg of protein/ml. DCs were subsequently activated with TNF-α and PGE_2 for 24 h.

Lysate-pulsed DCs Induce a Th1 Cytokine Profile in Cocultures with Autologous MNC. Nonadherent MNC cocultured with autologous DCs were weekly restimulated with DCs. DCs were either unpulsed or pulsed with lysate in the absence or presence of KLH. High levels of IL-12 were detected in the supernatants of MNC cocultured with mature DCs (Fig. 3). IL-12 secretion was enhanced in cultures in that antigens from tumor lysates were presented by DCs. A further increase was observed when KLH was added to the lysate. IFN-γ secretion was strongly dependent on the presentation of tumor antigens by DCs, again with higher levels detectable if KLH was added. In all of the cultures, IL-4 was near or below the detection limit.

T Cells Cultured with Lysate-pulsed DCs Up-Regulate CD69. CD69 expression is induced early in T-cell activation after stimulation of the T-cell receptor (29) and is expressed by antigen-specific T cells secreting IFN-γ and TNF-α (30, 31). From the same coculture as above, T cells were removed 48 h after the second stimulation with DCs, and the expression of CD69 was analyzed by flow cytometry. Whereas T cells cultured in the absence of DCs did not express this activation marker (data not shown), 2.9% of the CTLs ($CD8^+$) and 3.9% of the T-helper cells ($CD4^+$) expressed CD69 when cocultured with mature DCs (Fig. 4). Coculture with tumor lysate-pulsed DCs enhanced CD69 expression of both CTLs and T-helper cells to 8.3% and 9.4%, respectively. If KLH was added to the lysate, 17.1% of the CTLs and 17.8% of the T-helper cells expressed CD69.

T-Cell Proliferation Is Enhanced after Re-exposure to Tumor Antigens Presented by DCs. Assuming that lysate-pulsed DCs prime naive T cells toward tumor antigen-specific T cells and that these cells predominantly proliferate when exposed to antigen, we assessed the proliferation of MNC from the cocultures after restimulation with unpulsed or lysate-pulsed DCs. Incorporation of [^{3}H]thymidine into proliferating cells was assessed during the last 18 h of a 4-day coculture. MNC from the coculture with lysate-pulsed DCs showed an increase in proliferation rates after rechallenge with antigens presented by lysate-pulsed DCs compared with unpulsed DCs. If KLH was added to the lysate, a restimulation with lysate-pulsed DCs (no KLH) induced a further increase in proliferation (Fig. 5). Proliferation rates showed a good correlation with CD69 expression of T cells in all donors.

Lysate-pulsed DCs Induce a MHC Class I-restricted Tumor Cell Lysis. Next, we investigated whether MNC from the cocultures were able to specifically recognize and kill Panc-1 tumor cells. After four weekly stimulations with unpulsed, lysate-pulsed, or lysate- plus KLH-pulsed DCs, we removed the nonadherent cells from the cocultures and incubated them with ^{51}Cr-labeled Panc-1 tumor cells or KATO-III gastric carcinoma cells (HLA-$A2^-$). MNC from the coculture with lysate-pulsed DCs were able to specifically lyse Panc-1 tumor cells (Fig. 6). Lytic activity was enhanced in the cocultures with lysate- plus KLH-pulsed DCs. KATO-III was lysed to a minor extent only, and the lysis was comparable with that of Panc-1 by MNC that had been cocultured with unpulsed DCs. To test whether tumor cell lysis was MHC class I-restricted, we preincubated the tumor cells with a MHC class I-blocking antibody. This substantially reduced tumor cell lysis (Fig. 7). To exclude unspecific lysis mediated by NK cells, which contributed approximately 2–3% of the MNC at the end of the coculture, cytotoxicity directed against NK cell-sensitive K562 cells

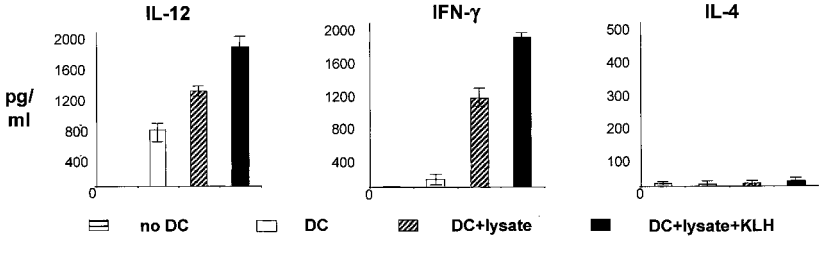

Fig. 3. DCs pulsed with Panc-1 tumor cell lysate induce a Th1 type cytokine profile. Forty-eight h after the second stimulation of MNC with unpulsed, lysate-pulsed, or lysate- plus KLH-pulsed DCs, supernatants were collected and analyzed for secreted IL-12, IFN-γ, and IL-4 by ELISA. Data are shown as means ± SE of three experiments.

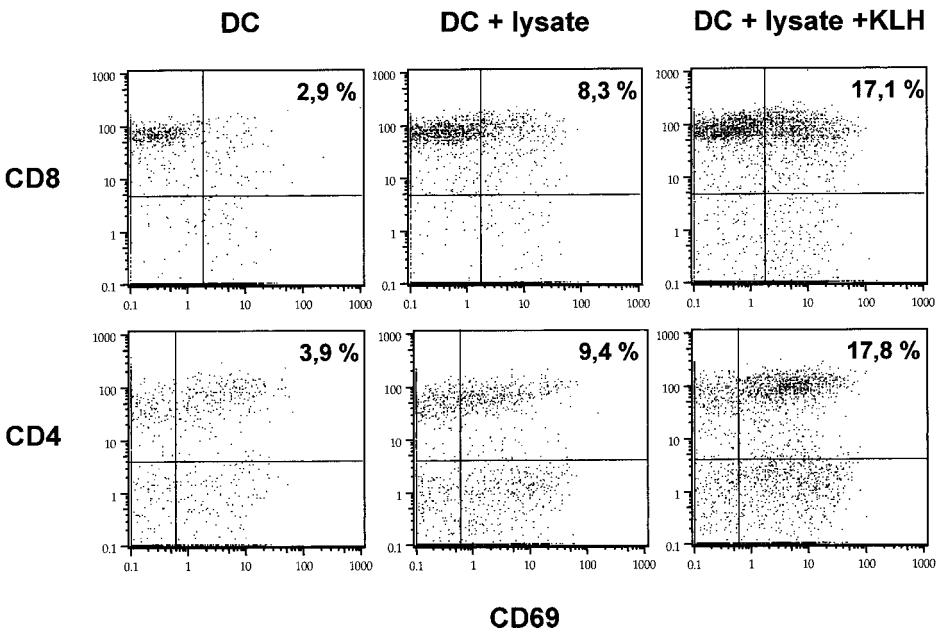

Fig. 4. Tumor cell lysate-pulsed DCs induce the expression of the activation marker CD69 by CTLs and by T-helper cells. Forty-eight h after the second stimulation by DCs, MNC were removed from the cocultures with unpulsed, lysate-pulsed, or lysate- plus KLH-pulsed DCs, and CD3-positive cells were analyzed for the expression of CD4, CD8, and CD69 by flow cytometry. A representative experiment of four is shown.

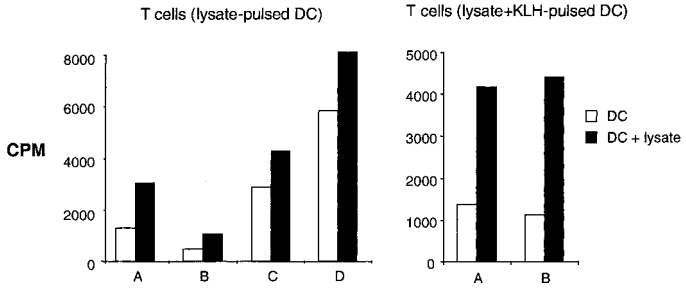

Fig. 5. T cells re-exposed to lysate-derived tumor antigens presented by DCs strongly proliferate. MNC from cocultures with tumor lysate-pulsed DCs (□) or lysate- plus KLH-pulsed DCs (■) were removed 7 days after the third stimulation and were cocultured with unpulsed or lysate-pulsed DCs. Proliferation was assessed by incorporation of [^{3}H]thymidine during the last 18 h of a 4-day culture.

was assessed, but no significant lysis could be observed (<5%; data not shown).

DISCUSSION

This study demonstrates the generation of an effective CTL response against pancreatic carcinoma cells by repeated *in vitro* stimulation of T cells with tumor lysate-pulsed DCs. Immature monocyte-derived DCs were used for pulsing with lysate because of their high phagocytic capacity (32). Subsequently, DCs were matured because DC-mediated immune responses are more effective if DCs receive an activation signal. This can be microbial products, such as lipopolysaccharide or unmethylated CpG motifs mimicking bacterial DNA (33), inflammatory mediators, such as TNF-α, PGE_2, IL-1β, IL-6, monocyte-conditioned media, and extracellular ATP (34, 35), or T cell-derived signals, such as CD40 ligand (36). Matured DCs up-regulate costimulatory molecules, secrete the T-cell differentiation factor IL-12, and present antigens more effectively because of increased phenotypic stability and extended half-life of MHC class I- and II-molecules (37). Furthermore, immature DCs bear the danger of inducing nonproliferating, IL-10-producing T cells, whereas mature DCs propagate the development of Th1 cells (38). In respect of clinical applicability, we activated DCs with a combination of TNF-α and PGE_2, which has been shown previously (39) to induce a mature phenotype with high expression of MHC class II, adhesion and costimulatory molecules, the secretion of IL-12, and enhanced T cell-stimulatory capacity.

Recently (40, 41), it has been postulated that DCs exposed to stressed or necrotic tumor cells mature spontaneously without the need of other activators. However, it cannot be excluded that this effect was caused by infection of the cell lines with *Mycoplasma* (42). In our hands, pulsing DCs with lysate from pancreatic carcinoma cell lines did not induce maturation as assessed by the lack of CD83 expression, but affected phenotype and T-cell-stimulatory capacity of DCs depending on the carcinoma cell line that was used. Enhanced T-cell stimulation of AsPc-1-pulsed DCs correlated with an increased surface expression of MHC class II and costimulatory molecules. Pulsing DCs with lysate of Panc-1 had no influence on surface marker expression or T-cell-stimulatory capacity, whereas lysate of Capan-1 completely inhibited DC-mediated T-cell proliferation. This inhibition did not correlate with the phenotype of the DCs and could not be reversed by subsequent activation with TNF-α and PGE_2. Because

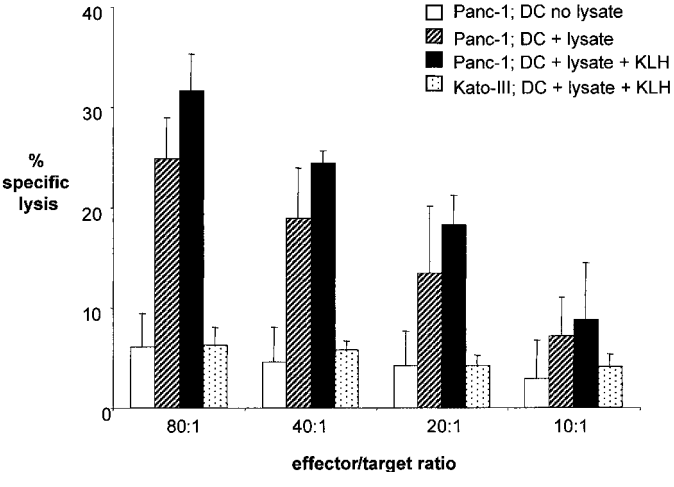

Fig. 6. Tumor lysate-pulsed DCs induce CTLs specific for Panc-1 carcinoma cells. MNC were removed from the cocultures with unpulsed, lysate-pulsed, or lysate- plus KLH-pulsed DCs and were incubated with ^{51}Cr-labeled Panc-1 tumor cells at the indicated ratios. The HLA-A2$^-$ gastric carcinoma cell line Kato-III served as a negative control. Data represent means ± SE of five independent experiments.

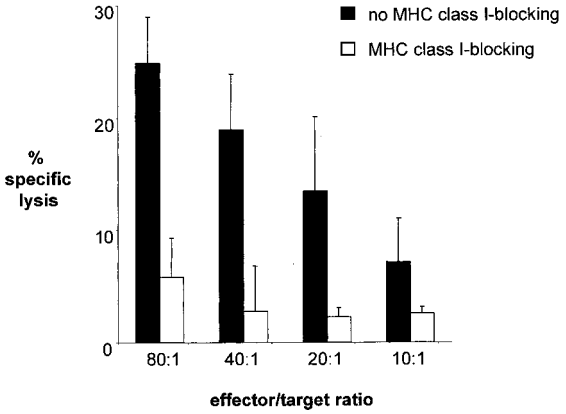

Fig. 7. Panc-1 tumor cell lysis is MHC class I-restricted. MNC from cocultures with lysate-pulsed DCs were transferred into wells with ^{51}Cr-labeled Panc-1 tumor cells at the indicated ratios. To prevent CTL-mediated cell lysis, the Panc-1 tumor cells were preincubated with the MHC class I-blocking antibody W6/32. Data are shown as means ± SE of five independent experiments.

viability and phenotype were not altered by Capan-1, soluble factors produced by the tumor cell line could be responsible for this dysfunction. It has been reported (43) that tumor cells produce factors impairing DC function, such as transforming growth factor β, IL-10, and vascular endothelial growth factor; transforming growth factor β reduces the allostimulatory capacity of DCs but does not influence the expression of MHC class II, CD54, or costimulatory molecules. IL-10 not only reduces the allostimulatory capacity of DCs, but also induces antigen-specific anergy of CTLs (44), whereas vascular endothelial growth factor inhibits maturation of DCs (45). Because functionally impaired DCs are less likely to induce effective CTL responses, we conclude that lysate-pulsed DCs should be monitored for their T-cell-stimulatory capacity before their use in clinical trials.

The cytokine profile in cocultures of MNC with lysate-pulsed DCs indicated the induction of a Th1 immune response with high levels of IL-12 and IFN-γ and low levels of IL-4. The increased levels of IL-12 in cultures with antigen-pulsed DCs can be explained by a positive feedback activation of DCs by antigen-specific T cells via mechanisms such as IFN-γ secretion and CD40-CD40L interaction (36). Only small amounts of IFN-γ were detected in the cocultures of MNC with unpulsed DCs compared with high amounts in the cocultures with lysate-pulsed DCs. Thus, IFN-γ secretion most likely reflects reactivity of T cells toward tumor antigens presented by DCs. IFN-γ secretion correlated with CD69 expression by both CD4 and CD8 lymphocytes in the cocultures. Activation of both CTLs and T-helper cells suggests that tumor antigens from lysates were presented via both MHC class I- and II-pathways.

To achieve clonal expansion of tumor antigen-specific T cells, the cultures were restimulated weekly with lysate-pulsed DCs. After the third stimulation, T-cell proliferation in response to tumor antigens was significantly enhanced, indicating an increased frequency of tumor-specific T cells in the cocultures. To determine whether T-cell activation and proliferation induced by DC coculture correlated with tumor cell lysis, we tested CTLs from the cocultures for their lytic activity against Panc-1 pancreatic tumor cells. After four stimulations, effective killing of Panc-1 was observed, whereas the HLA-A2$^-$ carcinoma cell line KATO-III was not lysed to a significant extent. The lytic activity appeared to be mediated by CTLs specific for Panc-1, because tumor cell lysis was MHC class I-restricted. However, this *in vitro* system does not allow defining the precise antigen specificity of the CTLs, and it cannot be excluded that other than tumor-specific antigens as well as additional MHC molecules could be involved in the recognition of the tumor cells. Unspecific lysis mediated by NK cells, contributing less than 3% to the MNC in the cocultures, is unlikely because no significant lysis of NK-sensitive K562 cells was observed. In conclusion, CTLs generated by repeated *in vitro* stimulations with lysate-pulsed DCs were able to specifically recognize and kill pancreatic carcinoma cells.

Adjuvants may play a major role in the induction of immune responses mediated by DCs (46, 47). The adjuvant properties of the highly immunogenic protein KLH have been used in vaccination trials either by coadministration of KLH with the DC vaccine (6, 9, 27) or by linkage of KLH to the relevant antigens (48–50). We observed that loading DCs concomitantly with lysate and KLH enhanced the T-cell response against tumor antigens. Higher levels of IL-12 and IFN-γ were detected in the supernatants, and an increased fraction of both CD4 and CD8 lymphocytes expressed the activation marker CD69. Furthermore, T cells from the cocultures with lysate- plus KLH-pulsed DCs proliferated to a higher extent when rechallenged with tumor antigens presented by DCs, and they lysed tumor cells more efficiently. These observations favor the concept that KLH can serve as a helper antigen augmenting tumor-specific immune responses (51). The simultaneous presentation of helper antigens and tumor antigens by DCs may lead to side-by-side activation of T cells specific for the helper and the tumor antigen, thereby providing a feedback signal for the antigen-presenting cell via CD40 ligation (36). The up-regulation of costimulatory molecules as well as secretion of IL-12 by the DCs could enhance the immunogenicity of tumor antigens that are otherwise not recognized by the immune system. Another mechanism involved could be a direct activation of CTLs by CD40-activated DCs (52). From our data, we conclude that KLH cannot be used only as a tracer molecule in vaccination trials, but also as an adjuvant for vaccines based on tumor lysate-pulsed DCs.

In summary, we demonstrated that CTLs specific for pancreatic carcinoma cells can be generated *in vitro* with tumor lysate-pulsed DCs. Vaccination of pancreatic carcinoma patients with lysate-pulsed DCs might offer an additional therapeutic option for these patients. Using autologous tumor cells for the generation of lysates may enable the immune system to generate a multiclonal T-cell response against a broad spectrum of tumor antigens expressed by the individual tumor. Furthermore, assuming that pancreatic carcinoma cells share common rejection antigens allogeneous carcinoma cell lines could be used as a source of antigen for patients who are not eligible for surgery (53). However, the feasibility of this approach still needs to be addressed in additional experiments.

We present an *in vitro* model applicable to improve and to monitor tumor vaccines based on DCs pulsed with unfractionated tumor antigens. We are currently investigating the influence of several parameters on the antitumor T-cell response such as maturational status of DCs, tumor antigen preparation (*e.g.*, unfractionated cell lysate, apoptotic tumor cells, apoptotic bodies, or tumor RNA), and the use of adjuvants to develop a DC-based tumor vaccine against pancreatic carcinoma.

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