Presentation of Renal Tumor Antigens by Human Dendritic Cells Activates Tumor-infiltrating Lymphocytes against Autologous Tumor: Implications for Live Kidney Cancer Vaccines

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ABSTRACT

The clinical impact of dendritic cells (DCs) in the treatment of human cancer depends on their unique role as the most potent antigen-presenting cells that are capable of priming an antitumor T-cell response. Here, we demonstrate that functional DCs can be generated from peripheral blood of patients with metastatic renal cell carcinoma (RCC) by culture of monocytes/macrophages (CD14+) in autologous serum containing medium (RPMI) in the presence of granulocyte macrophage colony-stimulating factor and interleukin (IL) 4. For testing the capability of RCC-antigen uptake and processing, we loaded these DCs with autologous tumor lysate (TuLy) using liposomes, after which cytometric analysis of the DCs revealed a markedly increased expression of HLA class I antigen and a persistent high expression of class II. The immunogenicity of DC-TuLy was further tested in cultures of renal tumor infiltrating lymphocytes (TILs) cultured in low-dose IL-2 (20 Biologic Response Modifier Program units/ml). A synergistic effect of DC-TuLy and IL-2 in stimulating a T cell-dependent immune response was demonstrated by: (a) the increase of growth expansion of TILs (9.4–14.3-fold; day 21); (b) the up-regulation of the CD3+CD56+ TcR+ (both CD4+ and CD8+) cell population; (c) the augmentation of T cell-restricted autologous tumor lysis; and (d) the enhancement of IFN-γ, tumor necrosis factor-α, granulocyte macrophage colony-stimulating factor, and IL-6 mRNA expression by TILs. Taken together, these data implicate that DC-TuLy can activate immunosuppressed TIL via an induction of enhanced antitumor CTL responses associated with production of Th1 cells. This indicates a potential role of DC-TuLy vaccines for induction of active immunity in patients with advanced RCC.

INTRODUCTION

RCC affects ~30,000 Americans per year (1, 2). Thirty % of patients have metastases at the time of diagnosis, and the prognosis is very poor, with a 1-year survival rate of 26%. Although a change in the natural history can be obtained by immunotherapy, e.g., IL-2 administration or autologous cellular infusion, the durable response rate is <20% (3, 4). Current treatment modalities lack specificity and are associated with significant toxicity (5). Innovative treatment options, therefore, are paramount.

DCs are the most potent cells for antigen presentation, are critical for eliciting T cell-mediated immune responses, and have initiated new directions for the treatment of cancer (6). The premise behind this approach is that DCs can be isolated from patients’ blood, armed with tumor antigens, and subsequently used to induce a specific antitumor response (7, 8). Recently, several human Phase I trials have been initiated using peptide-/protein-loaded DCs, and in different metastatic tumors, promising results have been obtained, without significant side effects from the vaccinations (9–11). It is known that a defective immune response exists in patients with advanced cancer (12, 13). Although factors that contribute to tumor immunological escape are not well elucidated, inadequate presentation of tumor antigens by host APCs is one of potential mechanisms that renders a tumor progression (14). To activate T-cell mediated antitumor activity, APCs must be capable of processing tumor-associated antigens, present them in the context of MHC class I or II molecules, and provide costimulatory molecules (B7-1/ B7-2) to interact with the T cell (15). The recent finding that mature DCs isolated from RCC tumors had a reduced capability...
of capturing soluble antigens indicate a close relationship between immunosuppression and defective DCs in situ (16). This hypothesis is further supported by the study demonstrating that pretreatment of DCs with transforming growth factor-β, a tumor-derived growth factor, could impair their antigen-presenting function (17). Lack of B7-1 and B7-2 expression by tumor cells was recently identified as an immunosuppressive factor that could block early stages of DC maturation from precursors (19). Therefore, the failure of RCC patients to elicit an antitumor response in situ, despite the presence of TILs and DCs, suggests that the antigen presentation system in situ in tumors is functionally suppressed. The epidemiological correlation between the number of tumor-infiltrating DCs and survival (20, 21) further indicates that activation of DCs within the tumor site may be particularly important in the induction of a cellular immune response.

In this study, therefore, we investigated the feasibility to generate functional DCs from PB-derived macrophages/moноcytes (CD14+) of patients with metastatic RCC. Because tumor growth is mainly regulated by the local immune status and, therefore, TILs, with their lack of immunoreactivity (22–24), reflect the tumor-host interaction more accurately than PBLS, we used the TIL culture system to test the tumor-specific immunosuppressive function of these DCs. Via the stimulation of these TIL cultures by autologous TuLy loaded DCs combined with IL-2 (20 BRMP units/ml), we attempted to elucidate TILs under immunosuppressive conditions by reestablishing an accurate antigen-presenting system in culture.

MATERIALS AND METHODS

Generation of DCs from PBLs. We adopted a technique for the culture of DCs previously described by Romani et al. (7), with slight modifications (25). Briefly, PBMCs were isolated from PB of patients with RCC by a Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech, Alameda, CA). Cells were resuspended in culture medium, either RPMI 1640 (BioWhittaker, Walkersville, MD) or AIM-V (Life Technologies, Inc., Gaithersburg, MD) supplemented with different concentrations of heparinized human type AB or autologous serum (0, 1, 5, or 10%), 0.01 M N-2-hydroxyethylpiperazine-N' ethanesulfonic acid buffer, and penicillin-streptomycin (50 units/ml; ICN Biomedical, Irvine, CA). Cells were plated in 25-cm² cultured flasks at 2.5 × 10⁶–5 × 10⁶ cells/ml. The cells were subsequently incubated at 37°C for 90–120 min, after which the nonadherent cells were removed with several gentle washes. Adherent cells were incubated at 37°C in complete medium supplemented with 800 units/ml human recombinant GM-CSF (Genetics Institute, Boston, MA) and 1000 units/ml human recombinant IL-4 (R&D Systems, Minneapolis, MN). After 7 days in culture, all free or loosely adherent cells were collected by vigorous rinsing. This resulted in a heterogeneous cell population containing DCs, B cells, T cells, and NK cells. DCs were further purified by negative depletion. The cell pellet was incubated for 30 min at 4°C with azide-free monoclonal anti-CD19, anti-CD3, and anti-CD56 antibodies (all from Immunotech, Westbrook, ME) at concentrations of 1 × 10⁶ cells per 2 μl of each. Antibody-labeled cells were removed using magnetic beads coated with goat antimouse IgG (Dynal, Lake Success, NY). To test the purity of the DCs, we also depleted these cells with anti-CD14 (Immunotech).

FACS Analysis. The phenotypic expression of DCs or TILs was determined by two-color fluorescence. Cells (5 × 10⁶) were resuspended in 50 μl of buffer (PBS, 2% newborn calf serum, and 0.1% sodium azide) and incubated with 10 μl of appropriate FITC- or PE-labeled mAbs for 30 min at 4°C. After incubation, the cells were washed twice and resuspended in 0.5 ml of assay buffer. The fluorescence was analyzed by a FACScan II flow cytomter (Immunocytometry System; Becton Dickinson, Mountain View, CA). Five thousand to 30,000 events were acquired for each sample using the FACScan Research Software that simultaneously acquires forward scatter, side scatter, FL1 (FITC label), and FL2 (PE label) data. The settings for all of these parameters were optimized at the initiation of the study and maintained constant during all subsequent analyses. Anti-HLA-DR (Immunocytometry System; Becton Dickinson), anti-HLA class I (W6/32, ATCC HB95), anti-CD14, anti-CD80 (B7-1; Caltag Laboratories, South San Francisco, CA), anti-CD86 (B7-2; PharMingen, San Diego, CA), anti-CD40 (Immunotech), and isotype control IgG1/IgG2a (Becton Dickinson) were used for characterization of DC phenotype. Anti-CD3, anti-CD56, anti-CD4, anti-CD8, anti-TcR (γ/δ), and control IgG1/IgG2a (Becton Dickinson) were used for characterization of the TIL phenotype.

Antigen Presentation Assay. Functional DCs were further defined by strong T-cell proliferative response to TT-pulsed DCs. From one of the patients, DCs were cultured, and from this same patient, T cells were purified. Briefly, TT-pulsed DCs or nonpulsed DCs were plated in triplicates in 96-well round-bottomed plates at concentrations of 1 × 10⁴, 2.5 × 10⁴, and 1 × 10⁵ cells per well per 100 μl of culture medium. Purified autologous T cells were then added to the wells at DC:T cell ratios of 1:10, 1:40, and 1:100, respectively. Cells were incubated for 6 days and pulsed with 1 μCi of [3H]thymidine (DuPont-NEN, Boston, MA) for an additional 24 h before harvesting (PhD Cell Harvester; Cambridge Technology, Cambridge, MA). The incorporated [3H]thymidine was measured by liquid scintillation counting. Results are shown as mean cpm of triplicates.

TuLy Pulsing of DC. RCC TuLy was prepared from 0–1–passage primary cell culture lines. Cells (5 × 10⁶) were harvested and resuspended in 200 μl of serum-free medium and were subjected to three freeze (dry ice)-thaw (37°C) cycles to obtain the crude lysate. The protein concentration of TuLy was determined by a protein assay (Bio-Rad DC₆, Protein Assay; Bio-Rad, Hercules, CA). TuLy delivery was carried out by incubating freshly cultured DCs with a mixture of TuLy and liposomes at a concentration of 30 μg of TuLy per 10⁶ DC. The liposomes (RPR GenCell, Palo Alto, CA) contain dimethyl-dioctadecyl-ammonium bromide (DDAB; cationic lipid) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; neutral lipid) in a 1:1 molar ratio. The TuLy was first mixed with liposomes at 1.5 % DDAB per 1 μg protein of TuLy. After an incubation of 20 min at room temperature, the complex was added to the DCs and incubated for 20 min at room temperature. After incubation, cells were diluted to 2× volumes with RPMI 1640 containing 20% human AB serum and further incubated at

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37°C and 5% CO₂ for 1 h. TuLy-pulsed DCs were extensively washed and then irradiated (3000 rad) before being used or stored at −80°C.

Cultures of TILs. Resected RCC tumors were immediately dissected under sterile conditions. The tumors were enzymatically digested by overnight stirring in sterile RPMI containing collagenase (type IV, 0.1%), hyaluronidase (type V, 0.01%), and DNase (type I, 0.002%; Sigma Chemical Co., St. Louis, MO). After Ficoll-Hypaque density gradient centrifugation (LSM, Organon Teknika, Durham, NC), the tumor-lymphocyte mixture was washed and seeded at concentration of 0.5 × 10⁶ cells/ml in culture plates supplemented with 20 units/ml (= 60 IU/ml) of recombinant IL-2 (Hoffman-La Roche, Inc., Nutley, NJ). TILs were stimulated with autologous TuLy-pulsed DCs (DC:TIL = 1:10) or TuLy alone twice weekly, 10–14 days after start of the TIL culture. Cell counts were performed every 5–7 days. Cells were split back to 5 × 10⁶ cells/ml after each cell count. TIL expansion was calculated by multiplying each expansion index (E.I. = (cell counts per ml)/(5 × 10⁶ per ml)). The cell concentration was readjusted with fresh IL-2-containing complete medium.

Capture of CD8+ TILs. The autologous CD8+ TILs were enriched from early cultures (day 10–14) of bulk TILs by positive selection with a anti-CD8+ mAb-coated culture flasks (CD8-CELLector; AIS Inc, Menlo Park, CA), and the purity of CD8+ cells was confirmed by flow cytometry analyses.

Cytotoxicity Assays. To enhance the detection of T cell-mediated cytotoxicity, the cytolytic activity of TILs was assayed for different target cells in a prolonged 51Cr release assay (18 h), as described previously (26). The assay was carried out in U-bottomed, 96-well microtiter plates. Target cells were prepared by incubating cells at 37°C for 1 h in the presence of 250 μCi of 51Cr/sodium chromate/10⁶ cells, washed four times, and counted. Five thousand target cells per well were mixed with TILs to yield several E:T ratios (40:1, 20:1, 10:1, and 5:1) and incubated for 18 h. Specific release of 51Cr was calculated by [specific 51Cr release = (experimental counts – spontaneous counts)/(maximal counts – spontaneous counts)] × 100%. To determine whether tumor lysis was T cell mediated, a blocking assay was performed in which TILs were pretreated with anti-CD3 or isotype control antibody for 30 min at 4°C prior to addition of cells to the cytotoxicity culture plates. Spontaneous release of tumor targets was <25%.

Quantitative PCR Analysis. Total RNA was extracted from cultured TILs 24 and 48 h after tumor restimulation by acid guanidine isothiocyanate-phenol-chloroform extraction. Reverse transcription of RNA to cDNA was performed by incubation of 2 μg of RNA with avian myeloblastosis virus reverse transcriptase using a oligo(dT) primer, dNTPs, and RNase inhibitor for 1 h at 42°C (cDNA Synthesis Kit; Boehringer-Mannheim Biochemica, Indianapolis, IN). One μl of each cDNA sample was amplified by PCR in a total volume of 25 μl using the specific primers against the cytokines IFN-γ, GM-CSF, TNF-α, IL-4, IL-6, and IL-10. The PCR mixtures were then amplified by undergoing 25 cycles in a DNA Thermocycler (Perkin-Elmer Corp., Norwalk, CT). Each cycle consisted of denaturation at 94°C for 1 min and annealing/extension at 65°C for 2 min. The 32P-labeled PCR products were visualized directly by acrylamide gel electrophoresis and autoradiography and then quantitated by excision of bands and subsequent scintillation counting. The signal intensity of each amplified product was calibrated to its corresponding β-actin mRNA expression as internal control for quantitation of expression levels. In addition, quantitative analysis was further elucidated by a serial dilution of mRNA (1:3, 1:10, 1:30, and 1:300) and coamplification of β-actin and cytokine mRNA.

RESULTS

Serum- and Cytokine-dependent Generation of DCs. During the period of the RCC study, blood was taken from 14 patients, and DCs were cultured. In one of the patients, the cultivation was unsuccessful because of contamination. With the addition of GM-CSF (800 units/ml) and IL-4 (1000 units/ml) in human AB serum containing RPMI medium, we were able to generate 1.2 × 10⁶ (± 0.3 × 10⁶) purified DCs from every 10 ml of PB, which corresponds to 5–12% of the PBMCs. We obtained a >95% purity of DCs, as characterized by phenotypic analysis (as previously described by our group; Ref. 25). The number of cells remained the same after negative depletion with anti-CD14, which proved at least a >95% CD14 negative population. The number of obtained DCs was lower (0.9 × 10⁶ ± 0.4 × 10⁶) when we used autologous serum. A representative phenotype of DCs derived from patients with RCC is presented in Fig. 1. FACS analyses revealed that DCs cultivated in RPMI plus 10% autologous serum supplemented with GM-CSF and IL-4 express high levels of B7-2 (Fig. 2b; MRLFI = 2912), B7-1 (MRLFI = 1669), HLA-DR (MRLFI = 7870), HLA class
I (MRLFI = 4120), and CD40 (MRLFI = 504). However, the CD14 expression on the cells was negative (MRLFI = 91). In addition, the cultured cells did not express lineage-specific markers for T (CD3), B (CD19), or NK (CD56) cells (data not shown). In contrast, DC cultivation in serum-free medium (AIM V) led to a relatively high percentage of CD14-expressing cells with reduced expression of B7-2 (Fig. 2, a, e, and f). Serum dependence of CD14 differentiation into DCs was further confirmed by the phenotype of cells generated in RPMI containing a reduced percentage of serum. An increase percentage of CD14 cells with low expression of B7-2 was detected when only 5% or no serum was used (Fig. 2, c and d). Unlike DCs generated in RPMI, failure of generation of APCs in AIM V medium cannot be rescued by supplementing of AIM V medium with serum. In our DC culture technique, the observation of serum- and medium-dependent generation of DC is consistent in several healthy donors (n = 3) and RCC patients (n = 2).

**Cytokine-cultured DCs Derived from PB of RCC Patients Are APCs.** To further characterize the function of antigen presentation by DC obtained under different culture conditions in our system, we determined their capability to induce proliferation of patients’ memory T cells in vitro against TT presented by autologous DCs. The highest T-cell proliferative response (35,000 cpm) was detected in T-cell cultures stimulated with DC that had been derived from GM-CSF plus IL-4 cultures, at a ratio of 1:10 (DCs:T cells). On the other hand, DCs cultured with GM-CSF alone exhibited a reduced stimulatory effect (12,500 cpm). No stimulatory effect was observed when nonloaded DCs or free TT was added (Fig. 3). To demonstrate antigen processing capacity by the cytokine-cultured DCs, we further compared the levels of HLA antigen expression in DC before and after liposome-mediated loading of RCC TuLy that was prepared from autologous tumor cells. Compared to nonloaded DCs, an enhanced HLA class I antigen expression was determined in DCs loaded with crude TuLy (MRLFI = 6834 versus 4120; Fig. 4), whereas HLA class II retained its high level of expression (MRLFI = 7870 versus 6825). No significant increase of expression of HLA I was observed when the DCs were loaded with liposomes without TuLy (MRLFI = 4855 versus 4120).
DC-mediated Growth Expansion of Autologous TILs. The induction of cellular immunity appears to be a crucial step for the development of antitumor activity. To evaluate the efficacy of RCC tumor vaccination using unfractionated autologous TuLy presented by DCs, we examined the ability of autologous TuLy-loaded DCs to stimulate a cellular immune response in RCC-derived TILs. When autologous DCs were loaded with RCC TuLy (DC:TIL = 1:10), which were cultured in low-dose IL-2 (20 BRMP units/ml), an enhanced growth expansion of TILs was observed. TIL expansion was calculated by multiplicity of each expanding index (E.I. = (cells counted per ml)/(5 × 10³ per ml)).

Table 1 Phenotypic modulation of TILs by DCs loaded with renal TuLy

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<th>Patient 2</th>
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<td>64 76 43</td>
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Values are percentages of TILs that are positive for a specific marker used in the cell staining. The cytometry analysis gates TILs based on their size and granularity. Isotype controls yl/2 were used to prove that the specific mAbs are not binding nonspecifically to TILs in the preparation. All TILs were grown in the presence of 20 BRMP units/ml IL-2. Culture stimulation was performed on days 7 and 14.

A, TILs stimulated with TuLy; B, TILs stimulated with DC-TuLy.

To identify which subsets of TIL were regulated by DC stimulation, we compared the phenotype of TILs stimulated with RCC TuLy (DC:TIL = 1:10), which were cultured in low-dose IL-2 (20 BRMP units/ml), an enhanced growth expansion (as measured by counting with trypan blue) of TILs determined in the four patients tested, compared to cultures stimulated with TuLy (mean = 22.3 ± 6.2 versus 9.2 ± 3.1).

DC-mediated Phenotypic Modulation of Patient Immune Cells. To identify which subsets of TIL were regulated by DC stimulation, we compared the phenotype of TILs stimulated with and without RCC TuLy-loaded DC (DC-TuLy) by FACS analysis. Compared to TILs stimulated with TuLy in the presence of low-dose IL-2 (20 BRMP units/ml), all four TIL cultures stimulated with DC-TuLy exhibited a decreased percentage of the CD3⁻CD56⁻ cell population (31, 14, 18, and 3% versus 48, 82, 40, and 15%, respectively), whereas an increased percentage of CD3⁺CD56⁻ cell population (46, 76, 75, and 80% versus 32, 23, 53, and 48%, respectively) with a predominant increase in the CD3⁺CD4⁺ cell population (46, 11, 63, and 66% versus 25, 3, 23, and 11%, respectively) was determined. A significant increased percentage of CD3⁺CD8⁻ cell population, however, was only observed in one of four TIL cultures (Patient 2, 56% versus 11%). An increase of TcR-positive cells was also determined in all DC-TuLy-induced TILs, compared to non-DC-stimulated cultures (44, 28, 76, and 76% versus 30, 2, 64, and 43%, respectively; Table 1). Because CD3⁺CD4⁻ cells were up-regulated by DC-TuLy, we asked, furthermore, whether DC-TuLy has any phenotypic effect on CD8⁻ cells. In these experiments, we used prepurified (>95%) CD8⁻ TILs for DC-TuLy stimulation. In comparison to cultures stimulated with TuLy, not only was a significant growth expan-
Dendritic Cells in Renal Cell Carcinoma

Table 2  Phenotypic modulation of purified CD8+ TILs by dendritic cells loaded with renal TuLy

<table>
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<td>CD3+CD8+</td>
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<td>17</td>
</tr>
<tr>
<td>CD3+TcR+</td>
<td>30</td>
<td>82</td>
</tr>
</tbody>
</table>

Values are percentages of TILs that are positive for a specific mAb used in the cell staining. TILs were grown in IL-2 for 1 week prior to CD8+ purification. Culture stimulation was performed on days 9 and 16.

... tion observed (data not shown) in the DC-TuLy-stimulated TILs but an induction of CD3+CD4+ (0 and 0% versus 12 and 17%, respectively) and CD4+CD8- (1 and 1% versus 17 and 13%) cells was also determined in both CD8+ TIL cultures tested (Table 2).

DC-TuLy-induced Tumor-specific Cytotoxicity of TILs

TILs grown under different conditions were tested for their killing activity against autologous RCC and allogenic RCC. After two cycles of restimulation with TuLy or DC-TuLy, DC-TuLy-induced TILs exhibited an augmented killing activity against autologous tumor target when compared to TuLy-induced TILs (Fig. 6a). However, a negligible cytotoxicity against allogenic RCC was determined (Fig. 6b). As a control, we used autologous TuLy alone (without DC), which did not show an outgrowth of a CTL. Subsequently, a blocking study was performed to elucidate this autologous killing was T-cell mediated. The blocking study was performed in two of the four patients, and a representative blocking (patient 3) is shown. In this patient, a marked reduction of cytotoxicity against autologous tumor was demonstrated in an assay in which effector cells (TILs) were pretreated with anti-CD3+ antibodies (Fig. 7). This effect was, however, limited when anti-CD8+ antibodies were used, which represents the percentage of CD4+ and CD8+ cells, as shown in Table 1.

The cytotoxic killing capacity of the TILs were also tested for two other cell lines: Daudi (NK insensitive) and K562 (NK sensitive) cell line. The percentage of cytotoxic killing (patient 1, 38% lysis; patient 2, 15%; patient 3, 21%; patient 4, 35% in the 40:1 E:T ratio) of the NK-sensitive cell line K562 by the TILs reflected the percentage of CD56+ cells in all four patients.

DC-TuLy Induces a Unique Cytokine Profile of TILs

To identify the cytokine mRNA profile of TIL that corresponds to increased proliferation and enhanced specific antitumor activity, we restimulated TILs and extracted RNA 24 and 48 h after stimulation in two patients. A typical cytokine mRNA expression profile is presented in Fig. 8 (similar for both patients). The data indicate that TILs grown under three different conditions (IL-2 alone, IL-2 plus TuLy, and IL-2 plus DC-TuLy) all expressed mRNAs of IFN-γ, GM-CSF, TNF-α, and IL-6 but not IL-4 and IL-10 (data not shown). However, the most enhanced expression of cytokine mRNAs was detected in TILs stimulated with DC-TuLy compared to non-DC-stimulated TIL cultures (IL-2 or IL-2 plus TuLy). RNA extraction 24 h after DC stimulation exhibits higher levels of IFN-γ (7-fold), TNF-α (2-fold), and IL-6 (3-fold) mRNAs when compared to RNA extracted 48 h after DC stimulation. In contrast to cultured TILs, no expression of IFN-γ, GM-CSF, or TNF-α mRNA was observed in noncultured TILs, indicating a sustained immune-suppressed status of these cells.

DISCUSSION

DCs play an important role in the immune system network as the most potent APCs (6). This unique function provides a fundamental concept and rationale for clinical applications in the treatment of patients with advanced cancer. Efficacy of peptide-pulsed DCs as APCs for the induction of antigen-specific CTLs in vivo has been demonstrated in a series of animal experiments using in vitro cultivated and cytokine-treated DCs (27–29). Experiments described in our research project provide evidence for a potent kidney cancer vaccine that may be generated using autologous TuLy-loaded DCs induced from adherent PBMCs using GM-CSF and IL-4. To develop a DC-based immunotherapy for the treatment of patients with RCC, we tried to optimize the DC culture technique suitable for clinical use. In our DC culture technique, the use of serum-free medium (RPMI or AIM V) was not suitable because it obtained a heterogeneous cell population with a significant number of CD14+ macrophages with a low B7-2 expression, a phenotype that is correlated with a lack of antigen uptake capacity and presenting function. Moreover, only with RPMI and 10% autologous serum were we able to generate adequate DCs from CD14+ progenitor cells (macrophage/monocytes; Fig. 1). A dose-dependent DC maturation by serum was only observed when DCs were cultured in RPMI medium but not in AIM V medium (Fig. 2). This medium- and serum-dependent DC maturation is obligatory in our culture technique in which, after the adherence step, the nonadherent cells are gently rinsed off and a significant number of lymphocytes are still present during the 7-day culture with IL-4 and GM-CSF. The combination of autologous serum and the presence of a small number of lymphocytes provide an adequate environment for monocytes to mature into potent APCs.

Finally, although GM-CSF is a crucial mediator of DC maturation and function, this method of DC maturation was also IL-4 dependent. In particular, the characteristic APC phenotype and function could only be demonstrated for DCs grown in the presence of both GM-CSF and IL-4 (Figs. 1 and 3).

In human RCC, T cell-defined epitopes have not yet been identified. Therefore, the use of TuLy as a source of tumor antigen is an attractive approach because it circumvents the requirement of identifying tumor-specific antigens (6, 30). The use of DCs loaded with tumor lysates has been explored in other tumors such as prostate carcinoma and sarcoma, and a T-cell proliferation was observed in vitro (31, 32). Moreover, mice vaccinated with DCs pulsed with unfractionated tumor peptides showed a reduced growth of s.c. established, weakly immunogenic tumors (33). The use of unfractionated tumor proteins, compared to a single peptide-loaded DC vaccination strategy,
may have advantages, although these advantages are still hypothetical. (a) Multiantigens loaded DCs may be able to present undefined but important epitopes, whereas approaches that use peptide-pulsed DCs require that the peptide is clearly defined. Therefore, multiantigen-loaded DCs are more likely to give multiple clonal expansion of T cells, which might have an enhanced antitumor effect. This effect has been shown in several human neoplasms, which have the capacity to elicit multiple specific immune responses in the autologous host (34). (b) Peptides processed by the DC from the unfractionated tumor proteins will mainly be presented in a MHC class II manner, inducing a CD4+ T-cell proliferation (32). Especially the induction of these T-helper 1 (Th1) cells has a proven stimulatory effect on the function of the DCs to induce CTLs (35, 36). Th1 cells express CD40L on the surface, which ligates to the CD40 expressed on DC and subsequently triggers the DC to produce extremely high levels of bioactive IL-12, a cytokine known to have a proliferative effect on CTL and an enhancement of the antigen-presenting function of the DC (35–37). Also other cytokines released by Th1 cells may give an environment neces-

Fig. 6 Cytotoxicity of TILs against autologous (a) and allogeneic (b) RCC tumor targets. Cytotoxicity activity of TILs was determined by 18-h 51Cr release assay that contained a titrated E:T ratio of 40:1, 20:1, 10:1, and 5:1. TILs were treated with two cycles of DC stimulation prior to assay. Spontaneous lysis for tumor target (5000 cells) was <25% of maximum lysis. ■, DC-TuLy-IL-2-stimulated TILs; ●, IL-2-stimulated TILs.
sary for further maturation of DCs. This may be similar to the effect accomplished by a recently described so-called monocyte-conditioned medium, which generates fully mature and stable DCs (38, 39). In our study protocol liposomes are used to load the TuLy on the DCs. Therefore, although, in general, the crude tumor extract-loaded DCs (surface-linked antigen) will lead to an MHC class II-restricted antigen presentation to CD4+ T, the cytoplasmic delivery of liposome-encapsulated antigen from an endocytic compartment allows the exogenous antigen to gain access to the class I presentation pathway (40, 41). Indeed, an up-regulation of HLA class I expression was detected in liposomal TuLy-loaded DCs compared to nonloaded DCs, whereas the high expression of HLA class II was sustained (Fig. 4). This suggests that the liposome-mediated antigen presentation in current preparations may occur via both HLA class I and class II pathways. Compared to non-DC stimulation, a down-regulation of NK cells with up-regulation of CD3+CD8+ was observed in all four DC-stimulated TIL cultures, which render a positive shift in the CD4+CD8+ ratio (Table 1). The TILs of patient 2, in which a significant outgrowth of CD3+CD8+ cells was observed, are one exception. Even in the purified CD8+ cells, this positive shift in the CD4+CD8+ ratio could be observed. In our in vitro studies, RCC-specific cytoxicity was detected in all cultures (Fig. 6). TILs grown in low-dose IL-2 expressed reduced cytotoxicity against autologous tumors compared to TIL costimulation with DC-TuLy. The addition of TuLy did not increase the cytotoxicity, suggesting a lack of activated APCs in the TIL culture. The demonstration of a lower cytotoxicity for both autologous and allogenic tumor targets (Fig. 6, a and b) in non-DC-cultured TILs further implicates a loss of cytotoxic T-cell growth in IL-2 alone, without DC stimulation. It appears that DC-TuLy-induced TILs do not represent single CTL clones because the phenotypic analysis revealed a heterogeneous cell population in the cultures. Also the cytokine profile of the restimulated TILs show patterns which correlates with tumor-specific lysis capacity of T cells (TNF-α and IFN-γ). Most likely, these cells represent multiple clones induced by multiantigens presented by DCs using unfractionated crude TuLy (Table 1, Fig. 8). In general, however, most of the autologous tumor lytic killing capacity of the DC-TuLy-stimulated TILs appeared to be cellular immunity mediated, as demonstrated by an almost complete disappearance of this capacity with anti-CD3 blocking (Fig. 7).

In summary, from the PB of patients with advanced RCC, we were able to obtain a significant number of DCs with an in vitro GM-CSF-/IL-4-based culture technique suitable for clinical use. These cells have a profound morphological and phenotypic entity and a functional capacity characteristic for potent APCs. When loaded with unfractionated tumor proteins using liposomes, these cells were able to induce, from TILs obtained from RCC tissue, a CTL with high autologous tumor cell-killing capacity. On the basis of the results obtained from this study, we proposed a DC-based vaccination strategy in patients with ad-
advanced RCC. At present such a trial (Investigational New Drug application no. 7121) has been designed and is ongoing at our institution.

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