ORIGINAL ARTICLE

Arch. Immunol. Ther. Exp., 2006, **54**, 403–409
PL ISSN 0004-069X

DOI 10.1007/s00005-006-0048-5

Anti-tumoral capabilities of effector cells after IFN-α or CpG-motif treatment of cocultured dendritic cells

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Received: 2006.05.17, **Accepted:** 2006.09.29, **Published online first:** 2006.11.21

Abstract

Introduction: *Ex vivo* expansion of monocyte-derived dendritic cells (mDCs) and subsequent coculture with autologous cytokine-induced killer (CIK) cells is an established system to create specific and non-specific anti-tumoral immunity. mDCs constitute the most frequently applied DC subset in clinical studies. One recently published approach to optimize the immunological functions of the DC/CIK cell system is the replacement of interleukin (IL)-4 by interferon (IFN)-α in the maturation process of the DCs.
Materials and Methods: The expressions of relevant surface antigens of IL-4-DCs and IFNα-DCs by flow cytometry and the anti-tumoral activation of effector cells cocultured with both types of DCs using cytotoxicity assays were compared. In addition, short-term coculture experiments with both types of DCs and IFNγ-LAK effector cells were performed and compared with standard CIK cell coculture experiments.
Results: Regarding the expressions of functionally relevant surface markers, no differences could be detected for CD80, CD83, and HLA-DR between IFNα-DCs and IL-4-DCs, whereas the mean fluorescence intensities of CD40, CD86, CD54, and HLA-ABC were decreased and the expression of CD14 was increased for IFNα-DCs. Moreover, no enhancement of cytotoxicity of cocultured CIK cells against tumor cell lines (A498 and SW480) was detected by the use of IFNα-DCs. Additionally, coculture experiments with IFNγ-LAK cells were performed and unexpectedly higher lysis rates in comparison with the established IL-4-DC/CIK coculture model was observed. Early incubation of the mDCs with several CpG-ODNs failed to increase the anti-tumoral cytotoxicity of the cocultured IFNγ-LAK cells.
Conclusions: These results demonstrate that in the mDC/CIK cell system, IFNα-DCs are not superior in inducing anti-tumoral cytotoxicity and even moderately inferior regarding the expression of functionally relevant surface markers compared with IL-4-DCs.

Key words: dendritic cells, CIK cells, LAK cells, interferon-α, interleukin-4, CpG-ODN.

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INTRODUCTION

Ex vivo expansion of monocyte-derived dendritic cells (mDCs) according to the protocol of Romani et al. [18] and subsequent coculture with autologous cytokine-induced killer (CIK) cells is an established system to create specific and non-specific anti-tumoral immunity *in vitro* and *in vivo* [23, 24]. CIK cells are a $CD3^+/CD56^+$ rich cytotoxic effector T cell population generated and expanded *in vitro* from peripheral blood cells. CIK cells have been shown to eradicate established tumors in a severe combined immunodeficiency (SCID) mouse lymphoma model [11] and have already achieved promising results in clinical studies [10, 21, 25]. Dendritic cells are capable of capturing and processing tumor antigens, expressing lymphocyte costimulatory molecules, and secreting cytokines and are therefore key tools to initiate specific anti-tumoral immune responses [17, 27]. Monocyte-derived DCs constitute the most frequently applied DC subset in clinical studies [12, 20] since they can be obtained easily from peripheral blood monocytes. Further optimization of the

immunological functions of this DC/CIK cell system to improve the efficiency of adoptive immunotherapy in the treatment of malignant diseases is highly desirable.

We used several approaches to optimize the DC/CIK cell system: One approach was to use interferon (IFN)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) instead of interleukin (IL)-4 and GM-CSF during the maturation process of mDCs. The literature provides inconsistent results concerning the replacement of IL-4 by IFN-α. Several authors report of a faster and more efficient differentiation of IFNα-DCs in terms of increased expression of functionally relevant surface markers (CD40, CD80, and CD86) and induction of T cell proliferation [2, 16, 19], whereas another study could not confirm these advantages of IFNα-DCs [1]. As far as we know, no comparison of the end-point cytotoxicity of effector cells stimulated with either IL-4-DCs or IFNα-DCs has been determined yet. We compared the expressions of functionally relevant surface antigens (CD14, CD40, CD80, CD83, CD86, CD54, HLA-ABC, HLA-DR) of IL-4-DCs and IFNα--DCs and the capacity of both mDC types to stimulate different effector cells in coculture experiments. Furthermore, we examined the effect of addition of several stimulatory CpG-oligodeoxynucleotides (ODNs).

MATERIALS AND METHODS

CpG-ODNs

Partially and fully phosphorothioate-modified CpG--ODNs were provided by Qiagen (Hilden, Germany). Sequences are shown 5´–3´; lower-case letters indicate phosphorothioate linkage and upper-case letters indicate phosphodiester linkage: ODN 2216: ggGGGA CGATCGTCgggggG, mockODN2216: ggGGGAGGA TGGTGgggggG, ODN 2006: TCGTCGTTTTGTCG TTTTGTCGTT, ODN 2395: TCGTCGTTTTCGGC GCGCGCCG. The CpG-ODNs were diluted in TE buffer (1 μg/ml) and frozen at –20°C until use.

Generation of mDCs (IL-4-DCs, IFNα-DCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by Ficoll density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The cells were adhered in 75-cm^2 culture flasks (Falcon, BD Biosciences Pharmingen, Heidelberg, Germany) at a density of 5×10^6 cells/ml for 1 h at 37°C in RPMI 1640 consisting of 10% autologous heat-inactivated serum, 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from PAA Laboratories, Cölbe, Germany). Suspension cells were collected for generating effector cells. The adherent cells were incubated in complete medium supplemented with 750 U/ml human GM-CSF and 500 U/ml human IL-4 (Essex Pharma, Nürnberg, Germany) or 1000 U/ml IFN-α (Roche Diagnostics, Mannheim, Germany), respectively, at a concentration of 1×10^6 cells/6-well. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and restimulated every third day with GM-CSF and IL-4 or IFN-α.

Incubation of DCs with CpG-ODN and lipopolysaccharide

DCs were pulsed on day 0 with 10 μg/ml ODN 2216, mock ODN 2216, ODN 2006, or ODN 2395. CpG-ODNs remained in the medium until the coculture was performed. As positive controls, IFNα-DCs and IL-4-DCs were exposed to 500 ng/ml lipopolysaccharide (LPS) for 24 h.

Generation of immunological effector cells

CIK and IFNγ-LAK cells were generated as described [6, 8, 23]. In brief, non-adherent Ficoll-separated human PBMCs were grown at a density of 3×10^6 cells/ml in complete RPMI 1640 medium containing 10% fetal calf serum (FCS). Both effector cell populations were exposed to recombinant human IFN-γ (Roche Diagnostics, Mannheim, Germany) (1000 IU/ml) on day 0 and recombinant human IL-2 (Roche Diagnostics, Mannheim, Germany) (300 IU/ml) on day 1. CIK cells were additionally stimulated with 50 ng/ml of Orthoclone OKT3 antibody against CD3 (Cilag GMBH, Sulzbach, Germany) and 100 IU/ml IL-1β. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and restimulated every third day with 300 IU/ml IL-2.

Coculture models

During long-term coculture, IFNα-DCs and IL-4-DCs were harvested either on day 4 or 6 and cocultured for a further 7 days with the respective effector cells in RPMI 1640 medium containing 10% FCS and 300 IU/ml IL-2. The cells were restimulated every third day by addition of 300 IU/ml IL-2 to the medium. During short-term coculture, IL-4-DCs and IFNα-DCs were harvested on day 1 and coculture was performed for 4 days with the respective effector cells in RPMI 1640 medium containing 10% FCS, 750 IU/ml GM-CSF, 300 IU/ml IL-2 and 1000 IU/ml IFN-α or 500 IU/ml IL-4. The cells were restimulated every third day by addition of 300 IU/ml IL-2 to the medium.

Target cell lines

A498 (human renal carcinoma cell line) and SW480 (human colon adenocarcinoma cell line) were maintained in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Flow cytometry/antibody staining

mDCs were harvested, washed, and resuspended in phosphate buffered saline supplemented with 1%

bovine serum albumin. After washing and incubation of 1×10^6 cells with different monoclonal antibodies (Pharmingen, Heidelberg, Germany or Beckman Coulter, Krefeld, Germany) conjugated to either fluorescein-isothiocyanate or phycoerythrin, the cells were analyzed by FACS (Coulter Epics XL cytometer). During FACS analyses, mDCs (on average 67.4±19.6%) were gated according to their expected physical properties. The portion of dead cells was evaluated by propidium iodide staining.

Cytotoxicity assay

The lysis capacity of effector cells was analyzed by a standard ^{51}Cr release assay against the two cancer cell lines. Target cell lines were labeled with ^{51}Cr and incubated with effector cells for 4 h at various effector/target (E:T) ratios. Supernatants were obtained and subjected to γ-counting (Top count NTX Packard, Meriden, USA). Maximum and spontaneous releases were respectively defined as counts from samples incubated with 1% IGPAL or medium. Cytolytic activity was calculated using the following formula:

% lysis=(experimental release – spontaneous release)×
×100/(maximum release – spontaneous release)

In all assays, spontaneous release was less than 20% of the maximum release and the maximum release was in excess of minimum 6300 counts per minute.

Statistical analysis

Statistical significance was determined by the 2-tailed Wilcoxon, Mann and Whitney U-test. Differences were considered statistically significant for $p<0.05$.

RESULTS

Comparison of IFNα-DCs and IL-4-DCs regarding expression of maturation-associated and functionally relevant surface antigens

Two different types of mDCs were derived from PBMCs. The first type (referred to as IL-4-DCs) was exposed to IL-4 and GM-CSF, whereas the second type (referred to as IFNα-DCs) was cultured in media containing IFN-α and GM-CSF. Both mDC types were treated with and without LPS 24 h before coculture on day 6 (IL-4-DCs) or on day 4 (IFNα-DCs). Previous studies have shown that IFNα-DCs develop a more efficient maturation process than do IL-4-DCs [5]. To verify these results we assessed the portion of cells expressing functionally relevant cell surface markers (%) and the corresponding average expression levels of these (mean fluorescence intensities – MFI). The following cell surface markers were analyzed: CD14, CD40, CD80, CD83, CD86, CD54, HLA-ABC, and HLA-DR. IL-4-DCs were examined on days 0, 4, and 6 with and without LPS treatment (Fig. 1A and 1C), whereas IFNα-DCs were evaluated on days 0, 1, and 4 with and without LPS treatment (Fig. 1B and 1D). As expected, both mDC populations showed no significant differences regarding the surface markers on day 0. On day 4, IFN-DCs showed an increased portion of CD14 positive cells compared with IL-4-DCs and significantly decreased MFIs of CD40 and CD86 (CD14: 16.75±7.05% vs. 0.82±0.73%, p=0.01; CD40: 40.55±8.60 vs. 126.52±41.02, p=0.05; CD86: 62.18±4.37 vs. 133.21±71.97, p=0.01). No difference between IFNα-DCs and IL-4-DCs could be observed with respect to the expressions of CD80, CD83, CD54, or HLA-DR.

Furthermore, we compared both types of DCs on the day of coculture, which was culture day 4 for IFNα-DCs and culture day 6 for IL-4-DC. The start of coculture two days earlier for IFNα-DCs than for IL-4-DCs was predefined since faster maturation of the IFNα-DCs was reported in the literature [5]. However, in our comparison, IL-4-DCs showed a more mature phenotype than IFNα-DCs: Significantly more IFNα-DCs expressed CD14 (CD14: 16.75±7.06% vs. 0.26±0.23%, p=0.01) compared with IL-4-DCs. Additionally, the MFIs regarding CD40, CD54, CD86, and HLA-ABC were significantly lower in IFNα-DCs than in IL-4-DCs (CD40: 40.55±8.55 vs. 249.74±72.67, p=0.01; CD54: 199.76±59.34 vs. 526.08±77.88, p=0.01; CD86: 62.18±4.37 vs. 118.24±2.81, p=0.02; and HLA-ABC: 41.05±17.10 vs. 110.34±49.33, p=0.01). No significant difference between IFNα-DCs and IL-4-DCs in the expressions of CD80, CD83, or HLA-DR was observed on the day of coculture.

Similarly, IFNα-DCs showed a less mature phenotype and seemed to be less inducible by LPS (24 h treatment). The portion of CD14-expressing cells was significantly higher and the proportions of CD40-, CD86-, and CD54-expressing cells were significantly lower compared with those of IL-4-DCs (CD14: 11.14±6.95% vs. 0.53±0.71%, p=0.01; CD40: 97.28±1.68% vs. 99.48±0.71%, p=0.02; CD86: 97.84±2.06% vs. 99.47±0.27%, p=0.02; CD54: 98.58±1.08% vs. 99.90±0.13%, p=0.01). Moreover, the MFIs of CD40, CD54, CD86, and HLA-ABC were significantly decreased in IFNα-DCs compared with IL-4-DCs (CD40: 47.64±19.65 vs. 362.65±108.02, p=0.01; CD54: 337.49±132.70 vs. 1309.49±414.66, p=0.01; CD86: 153.82±126.51 vs. 193.74±41.75, p=0.05; HLA-ABC: 38.96±0.16 vs. 108.84±27.80, p=0.01). We could not detect any significant difference regarding the expressions of CD80, CD83, or HLA-DR after LPS stimulation.

In conclusion, IFNα-DCs presented a moderately less mature phenotype than did IL-4-DCs. Regarding the proportion of necrotic cells, no differences were found between IFNα-DCs and IL-4-DCs. The complete marker expression profiles of both DC types during the culture are shown in Fig. 1A–D.

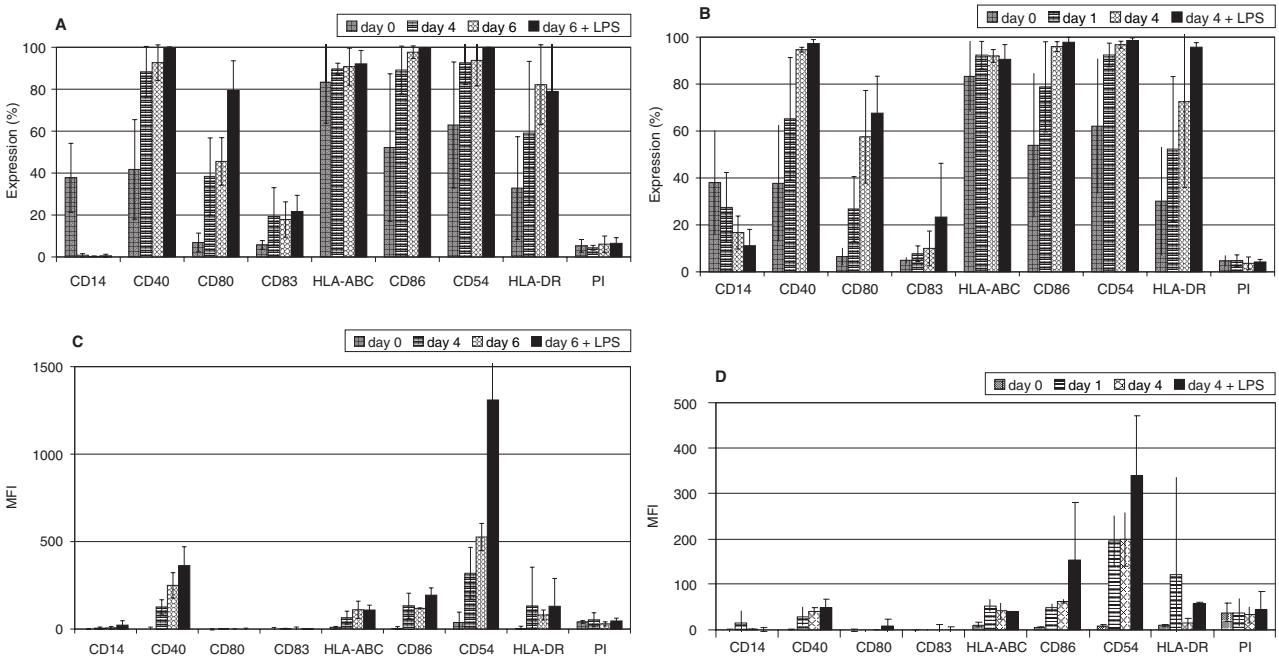

Fig. 1. Maturation profile of IL-4-DCs and IFNα-DCs. **A** – maturation and immunostimulatory markers of IL-4-DCs. Time course of maturation and immunostimulatory markers of IL-4-DCs. On culture days 0, 4, 6, and 6 plus LPS, expression (%) of the following markers was analyzed by FACS: CD14, CD40, CD80, CD83, HLA-ABC, CD86, CD 54, and HLA-DR. The portion of dead cells during culture time was assessed by propidium iodide (PI) staining. Six independent experiments were performed. **B** – maturation and immunostimulatory markers of IFNα-DCs. Time course of maturation and immunostimulatory markers of IFNα-DCs. On culture days 0, 1, 4, and 4 plus LPS, expression of the following markers was analyzed by FACS: CD14, CD40, CD80, CD83, HLA-ABC, CD86, CD 54, and HLA-DR. The portion of dead cells during culture time was assessed by PI staining. Six independent experiments were performed. **C** – MFI of maturation and immunostimulatory markers of IL-4-DCs**.** Time course of MFI of maturation and immunostimulatory markers of IL-4-DCs. On day 0, 4, 6, and 6 plus LPS expression of the following markers was analyzed by FACS: CD14, CD40, CD80, CD83, HLA-ABC, CD86, CD 54, and HLA-DR. Six independent experiments were performed. **D** – MFI of maturation and immunostimulatory markers of IFNα-DCs. Time course of MFI of maturation and immunostimulatory markers of IFNα-DCs. On day 0, 1, 4, and 4 plus LPS, expression of the following markers was analyzed by FACS: CD14, CD40, CD80, CD83, HLA-ABC, CD86, CD 54, and HLA-DR. Six independent experiments were performed.

Comparison of IFNα-DCs and IL-4-DCs regarding promotion of cytotoxicity of CIK cells following standard coculture

IL-4-DCs and IFNα-DCs were harvested and cocultured with CIK cells for a further seven days. For these experiments, cells of the above-described FACS-phenotyped DC-preparations were used. The lysis capacity of the effector cells was analyzed by a standard ^{51}Cr-release assay. A renal carcinoma cell-line (A498) and a colon cancer cell-line (SW480) served as target cell lines. Concerning cytotoxicity, no differences could be detected between IFNα-DC- and IL-4-DC-cocultured CIK cells for both target cell lines. Figures 2A and 2B show details.

Comparison of IFNα-DCs and IL-4-DCs regarding promotion of cytotoxicity of IFNγ -LAK cells

To put the comparison of IFNα-DCs and IL-4-DCs into a broader context, we additionally applied IFNγ-LAK cells as alternative effector cells and tested the cytotoxicity promoted by both DC types. During this short-term coculture, both DC types were incubated only one day alone and subsequently cocultured with autologous IFNγ-LAK effector cells for 4 days. For continued maturation, the GM-CSF and IL-4 or IFN-α mDCs were maintained in medium. A renal carcinoma cell-line (A498) served as the target cell line. Again, and comparable to the results of the standard-term cultures, no significant differences in anti-tumoral cytotoxicity of effector cells cocultured either with IFNα-DCs or IL-4-DCs could be detected (Fig. 2B). Surprisingly, IFNγ-LAK effector cells from both the IL-4-DC and IFN-DC short--term cocultures exhibited significantly increased tumor cell lysis at several effector-to-target ratios compared with the corresponding CIK effector cells from the standard coculture model (Fig. 2B).

Incubation of DCs with CpG-ODN

In an additional attempt to increase the anti-tumoral cytotoxicity of effector cells, we incubated mDCs (PBMCs) on day 0 not only with the cytokine cocktail, but also with various CpG-ODN (ODN 2216, mock ODN 2216, ODN 2006 and ODN 2395) and LPS. After

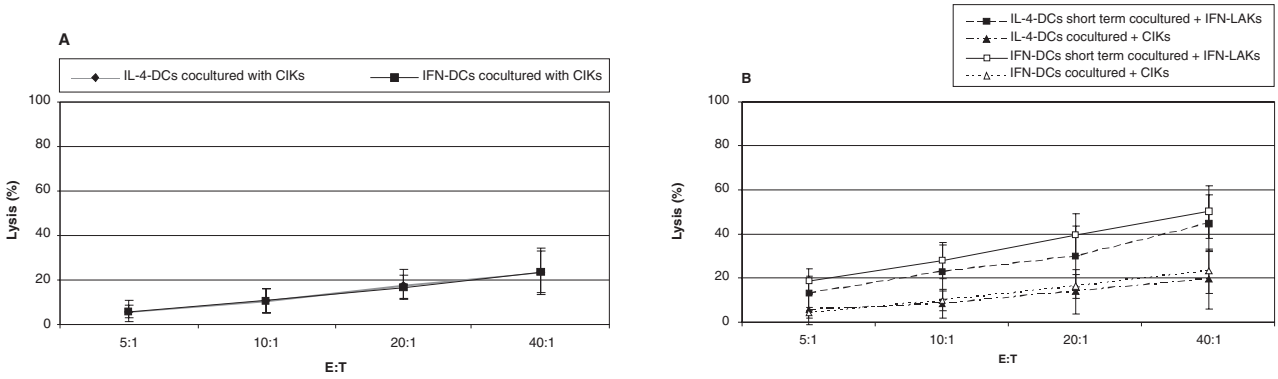

Fig. 2. Cytotoxicity of effector cells after coculture with IL-4- and IFNα-DCs. **A** – cytotoxicity against SW480 of CIK cells standard-term cocultured with IL-4-DCs and IFNα-DCs. CIK cells were cocultured for 6 days with autologous IL-4-DCs or IFNα-DCs on day 6 or 4, respectively. After coculture the cytotoxicity of CIK cells against SW480 cells was tested by standard ^{51}Cr-release assay. No significant differences could be observed in six independent experiments for both mDC types. **B** – cytotoxicity of standard-term cocultured CIK cells vs. cytotoxicity of short-term cocultured IFNγ-LAK cells, both cocultured with IL-4-DCs or IFNα-DCs**.** CIK cells were cocultured for 6 days with autologous IL-4-DCs or IFNα-DCs which had been previously cultured for 6 days or 4 days, respectively. IFNγ-LAK cells were cocultured for 4 days with autologous IL-4-DCs or IFNα-DCs which had been previously cultured for 1 day. After coculture, cytotoxicity of effector cells against A498 cells was tested by ^{51}Cr-release assay. No significant differences were observed when IL-4-DC-based experiments were compared with IFNα-DC-based experiments. However, short-term IFNα-DC-cocultured IFNγ-LAK cells showed a significantly higher lysis rate than the IFNα-DC-cocultured CIK cells. IFNγ-LAK cells short-term cocultured with IL-4-DCs showed a significantly increased lysis rate compared with IL-4-DC-cocultured CIK cells.

analyzing the cytotoxcity of cocultured IFNγ-LAK cells against A498, no significant differences could be found. IL-4-DC cytotoxicity (E:T 40:1) without ODNs was 44.75±12.75 and with the ODNs 2216, 2216 mock, 2006, or 2965 they were 41.04±13.75, 46.43±9.53, 40.07±9.63, and 37.75±10.53, respectively. IFNα-DC toxicity without ODNs (E:T 40:1) was 50.09±11.98, and the respective values with an ODN were 51.13±10.75, 44.67±10.78, 42.24±15.63, and 52.09±9.67 (n=10 in experiments without ODN, n=6 in experiments with ODN).

DISCUSSION

Ex vivo expanded CIK cells stimulated by autologous mDCs represent advanced tools of immunotherapy against cancer. mDCs are well known to activate CIK cells both in antigen-specific [13, 14] and unspecific ways [15]. Several clinical studies have been performed recently [10, 21, 25]. Therefore we felt that continued examination of modifications of the conventional DC/CIK cell system was required to improve its efficiency. First, we examined whether replacement of IL-4 by IFN-α during the culture could induce a more mature immunophenotype of mDCs. However, we observed no increase in expression of several functionally relevant cell surface markers (CD80, CD83, HLA-DR) in IFNα-DCs, and even a decrease in expression of other markers (CD40, CD86, CD54, HLA-ABC) at some stages of culture and an increased portion of mDCs which remained CD14 positive. Moreover, no enhancement of cytotoxicity of cocultured CIK cells against tumor cell lines (A498 and SW480) was detected. This lack of induction of increased cytotoxicity by IFNα-DCs was confirmed when IFNα-DCs and IL-4-DCs were compared after use of another type of effector cells (IFNγ-LAK cells).

The literature provides contradictory results regarding the utility of IFN-α for stimulation of mDCs. IFNα-DCs have been recently reported by several groups to show increased expressions of CD40, CD80, CD83, and CD86 compared with conventionally IL-4-stimulated mDCs [2, 3, 19]. In contrast, Dauer et al. [1] could not confirm the superiority of IFNα-DCs, but found that mDCs derived from IFN-α-treated monocytes are even defective in maturation, IL-12 secretion, and T cell stimulatory capacity. The results of our analyses are more consistent with the results of Dauer et al. [1] than with the results of the authors who observed a superiority of IFNα-DCs. The reasons for the lack of consensus in the literature are unclear. One possible explanation may be the use of different type I interferons. We applied IFN-α 2a, Della Bella et al. [2] used IFN-α 2b, Santini et al. [19] used consensus IFN, and Dauer et al. [1] did not specify the subtype of the IFN-α used. Moreover, it is a matter of debate on which day of culture the different DC types should be compared. Dauer et al. [1] considered the data of Santini et al. [19] as not conclusive since the latter authors performed the comparative examinations early, on day 3 of DC culture, compared with day 6, as Dauer did. We observed no superiority of IFNα--DCs even on the relatively early culture day 4.

To our knowledge, we present here the first comparative cytotoxicity assays of effector cells stimulated by either IFNα-DCs or IL-4-DCs. We could not observe any difference in anti-tumoral cytotoxicity of CIK effector cells cocultured with IFNα-DCs compared with CIK

effector cells cocultured with IL-4-DCs. In a further coculture approach, another type of effector cells (IFNγ-LAK) was used. Surprisingly, we observed markedly higher lysis rates of up to 50% (E:T ratio: 40:1) in these shorter IFNγ-LAK coculture experiments compared with the established DC/CIK coculture model. A possibility to shorten the duration of DC and effector cell cultures would be a considerable advantage in terms of economy and risk of microbial contamination.

CIK cells have been reported to have an enhanced cytotoxicity against lymphoma and leukemia cell lines [22, 23] and to have superior *in vivo* anti-tumor effects in a SCID mouse model of human lymphoma compared with standard LAK cells [11, 24]. However, CIK cells have been less intensively investigated and compared with LAK cells in solid tumors. Wang et al. [26] showed that CIK cells originating from patients with hepatocellular carcinoma (HCC) possessed a higher *in vitro* antitumor cytotoxic activity on autologous HCC cells than the autologous LAK cells, but, to our knowledge, no comparisons between CIK and LAK cells have been performed in more frequent solid tumors such as renal cell carcinoma or colon carcinoma. Our results demonstrate that in these tumor cells, IFNγ-LAK cells may be more effective than CIK cells and that it is recommended to compare different effector cell populations during preparation phases of new adoptive immunotherapy studies.

In further experiments, we tested the potential role of CpG-ODN in the stimulation of IL-4-DCs and IFNα-DCs. Early incubation (culture day 0) of DCs with several CpG-ODNs did not result in an increase in anti-tumoral cytotoxicity of cocultured IFNγ-LAK cells. This is most likely explained by a Toll-like receptor (TLR)9-dependent unresponsiveness of mDCs to CpG-ODN and extend the findings of our previous study [4]. In that study we did not observe any increase in the immunological capabilities of mDC after CpG exposure during standard-term culture. However, we considered it necessary to reevaluate this approach in the short-term DC culture since TLR9 mRNA has been detected in monocytes [7], which represent a larger portion of the adherent cells in the early incubation phase of our short-term culture experiments. Furthermore, our results support other authors who regarded mDCs as CpG-resistant [9].

In conclusion, our results demonstrate that: 1) in our models, IFNα-DCs are similar but not superior to IL-4-DCs, 2) CpG-ODNs do not increase the potential of early mDCs to induce cytotoxicity of short-term cocultured IFNγ-LAK cells, and 3) short-term coculture protocols applying IFNγ-LAK cells may be more cytotoxic than conventional CIK cells in certain solid tumor cell lines. These findings might be valuable for the design of future studies of adoptive anti-tumoral immunotherapy.

Acknowledgment: This work was kindly supported by the H.W. & J. Hector Stiftung, Weinheim, Germany.

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