ORIGINAL ARTICLE

Angelica Loskog · Christina Ninalga
Thomas H. Tötterman

Dendritic cells engineered to express CD40L continuously produce IL12 and resist negative signals from Tr1/Th3 dominated tumors

Abstract TNFα-matured dendritic cells (DCs) pulsed with tumor antigens are being evaluated as cancer vaccines. It has been shown that DCs produce IL12 during a limited time span and subsequently enter a stage of IL12 exhaustion. If DCs are generated ex vivo, the patient could receive IL12-exhausted DCs which may be detrimental for stimulating anti-tumor Th1 responses. Furthermore, many cancer patients exhibit a cytokine profile skewed toward IL10 and TGFβ. This immunological profile, called the Tr1/Th3 response, is associated with the presence of regulatory T-cells. Tr1/Th3 responses potently inhibit DC maturation, thereby regulating Th1 responses. In the present study, we produced genetically engineered DCs that continuously express Th1-related cytokines such as IL12, and resist negative signals from Tr1/Th3-dominated bladder carcinoma cells. Human immature DCs were genetically engineered by adenoviral vectors to express CD40L, or were treated with TNFα as a positive control for maturation. The expression of different Th1/Th3 and inflammatory cytokines was monitored. IL12 and IFNγ were expressed by CD40L-engineered DCs, while TNFα-matured DCs lacked IFNγ and exhibited low IL12 expression. The addition of recombinant IL10 to genetically engineered DCs did not abolish their Th1 profile. Likewise, coculture with tumor cell lines expressing TGFβ with or without recombinant IL10 did not revert to the engineered DCs. We further demonstrate that the resistance of CD40L-expressing DCs to TGFβ and IL10 may be due to decreased levels of TGFβ and IL10 receptors. Thus, CD40L-engineered DCs are robust Th1-promoting ones that are resistant to Tr1/Th3-signaling via IL10 and TGFβ.

A. Loskog (✉) · C. Ninalga · T. H. Tötterman
Clinical Immunology Division, Rudbeck Laboratory,
Uppsala University, Dag Hammarskjoldsvag,
20 751 85 Uppsala, Sweden
E-mail: angelica.loskog@klinimm.uu.se
Tel.: +46186119181
Fax: +46186110222

Introduction

The immune system has the capacity to eradicate tumor cells by several mechanisms. Anti-tumor responses are generated along the so-called Th1 pathway driven by IL12 and IFNγ production [1]. The most potent immunotherapeutic strategies against cancer engage the activation of tumor-specific cytotoxic T cells (CTLs) [2]. Tumor-specific CTLs are detectable in cancer patients, but are often sparse, anergic and unable to control the growing tumor. Anergy or different stages of unresponsiveness are induced by several mechanisms including antigen presentation in the absence of costimulation [3].

Key players in the activation and maintenance of CTLs are dendritic cells (DCs). These cells are superior antigen presenting cells (APCs) due to their high expression of major histocompatibility complex (MHC) molecules and because they supply important adhesion and costimulatory molecules to lymphocytes [4, 5]. Immature DCs are efficient antigen-capturing cells. Dendritic cells respond to environmental signals such as CD40L from T helper cells, TNFα, heat-shock proteins, and bacterial components which affect their differentiation into mature DCs. Upon maturation, their capacity for antigen capture decreases and, instead, they exhibit several properties necessary for processing and presenting captured antigens with high efficiency [6]. Mature DCs upregulate their secretion of immune stimulating cytokines such as IL12, and enhance their cell surface expression of costimulatory molecules like B7.1/2 [4]. Immature DCs and regulatory T-cells present in the tumor area may be responsible for the inactivation of CTLs. The role of regulatory T-cells has been intensively investigated over the past few years. They have been detected in several different malignancies either in the

tumor area or at elevated levels in patient blood [7–9]. Regulatory T-cells need antigens for activation and proliferation but unlike conventional T cells they act in an antigen-independent fashion. They have been subdivided according to a cytokine pattern consisting of IL10, TGFβ, IL4, and IFNγ alone or in different combinations (Tr1/Th3) [10]. It has been shown that IL10 and TGFβ inhibit DC maturation and may thereby indirectly eliminate CTL responses [11]. They are also known to inhibit Th1-related immune responses while favoring Th2 or regulatory responses (Tr1/Th3). Further, IL10 is considered a common immune escape mediator since tumor growth is often associated with this molecule [3, 12]. Seo et al. showed, in a murine B16 melanoma model, that IL10 expressed early in the tumor lesion was critical for the generation of CD4 + regulatory T-cells which inhibited CTL activity [13].

In standard protocols of DC generation, DCs are differentiated from monocytes cultured with IL4 and GM-CSF. To further differentiate the immature DCs to exhibit a mature phenotype with enhanced antigen presentation and costimulation, DCs are incubated for 24 h with TNFα. These DCs are known to express IL12, albeit for a short time span of 8–16 h. Hence, ex vivo generated DCs pulsed with tumor antigens may be introduced to the patient in an IL12-exhausted state. Clinical trials have shown that patients display only a minor to moderate increase in anti-tumor −specific T-cell responses, with most patients exhibiting progressive disease. Still, selected patients have shown a stable disease pattern or partial responses even in advanced cancer [14–16]. With optimized protocols for DC generation and stimulation, DC vaccination may have considerable potential in the treatment of cancer.

In the present study, DCs were genetically engineered to express CD40L in order to make them resistant to the inhibitory effects of Tr1/Th3 cytokines such as IL10. These DCs were not only resistant to IL10, but also opposed negative signals including TGFβ expression from Tr1/Th3-dominated bladder carcinoma. Furthermore, we demonstrate that DCs, upon constant CD40L stimulation, provide long-lasting IL12 response important for driving potent Th1 anti-tumor responses.

Materials and methods

Cell culture and DC generation

Human bladder cancer cell lines RT-112, KV-19-19 and VM-CUB-1 were obtained from DSMZ, Braunschweig, Germany, while the T24 cell line was a kind gift from Dr. A. Richter Dahlfors, Karolinska Institute, Stockholm, Sweden. T24, RT-112 and KV-19-19 were cultured in RPMI-1640 supplemented with 10% FBS and 1% Penicillin–Streptomycin while VM-CUB-1 was cultured in DMEM with the supplements mentioned previously, and 0.1% sodium-pyruvate (InVitrogen,

Paisley, Scotland). Human monocytes were obtained by CD14 + selection (90–98% purity) using Magnetic Cell Sorting (MACS) (Miltenyi Biotech GmbH, Gergisch Gladbach, Germany). Monocytes were differentiated into DCs by 7 days of stimulation with 50 ng/ml GM-CSF and 25 ng/ml IL4 (RPMI-medium stated above).

Adenoviral vectors

Vectors were constructed and produced as described previously [17]. Briefly, CD40L cDNA was inserted into the AdEASY adenoviral vector system (kind gift from Dr. B. Vogelstein, Johns Hopkins, Baltimore, MD). E1 and E3-deleted, replication deficient recombinant adenoviruses were produced by four rounds of infection of 293 cells. Virus titers were determined by plaque assay.

Adenoviral vector transduction

Adenoviral vectors AdCD40L and AdlacZ (gift from Canji Inc., San Diego, CA) were used to transduce cells in vitro. DCs and cell lines were transduced with 100–200 PFU/cell with the afore-mentioned vectors. Briefly, cells were washed in PBS, centrifuged and resuspended in 200 μl DMEM or RPMI without supplements. Adenoviral vectors were added and incubated with the cells for 2 h at 37°C. A DMEM or RPMI medium was then added and cells were transferred to 6-well plates. The transduction efficiency was 50–80% transgene-expressing cells at day 3 post-transduction. Transduction was well-tolerated with respect to cell viability and negligible cell toxicity was observed.

In vitro stimulation and evaluation of DCs

One million immature DCs from three healthy donors were transduced with adenoviral vectors or stimulated with recombinant TNFα (40 ng/ml) and cultured in a total volume of 3 ml RPMI medium in 12-well plates. Recombinant IL10 was added in some groups in two dosages: 2.0 and 20 ng/ml. The total supernatants were harvested at three time points: days 2, 3 and 7 whereupon a new medium with TNFα or IL10 was added. Recombinant cytokines were purchased from Nordic Biosite, Stockholm, Sweden. Viable human bladder cancer cell lines were mixed with immature DCs in an RPMI medium in a 1:5 ratio (3×10^5 total cells) in a total volume of 3 ml in 24-well plates. The coculture was continued for 3 days and subsequently harvested and analyzed by flow cytometry using antibodies directed against MHC class II, CD80, CD83, CD40L and IL10R (BD Biosciences, San Diego, CA). For detection of TGFβR-I, -II and -III, the DCs were cytospun onto glass slides and stained by immunocytochemistry. The following primary antibodies were used: rabbit anti-

human TGFβR-I (polyclonal), mouse anti-human TGFβR-II (monoclonal) and goat anti-human TGFβR-III (polyclonal) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). For detection of TGFβR-I and -II, ENVI-SION systems for rabbit and mouse were used (Dako-Cytomation, Denmark). For detection of TGFβR-III, a rabbit anti-goat Ig was used as a secondary step together with Goat PAP (DakoCytomation, Denmark). The supernatants were harvested and analyzed by cytometric bead array for cytokines Th1/Th2 Kit (IL2, IL4, IL6, IL10, IFNγ and TNFα), Inflammation Kit (IL1, IL6, IL8, IL10, IL12p70 and TNF) (BD Biosciences, San Diego, CA) and IL12p40 ELISA (Nordic Biosite, Stockholm, Sweden).

Quantitative PCR

RNA was isolated using TRIzol reagent and cDNA was obtained with Superscript II Reverse Transcriptase (InVitrogen, Paisley, Scotland) using random hexamer primers (CyberGene AB, Stockholm, Sweden). Quantitative PCR was performed using the TaqMan system (iCycler, Bio-Rad Laboratories, Hercules, CA). The cDNA was mixed with PCR Gold Buffer, Taq Polymerase AmpliTaq Gold (Applied Biosystems, Foster City, CA), MgCl$_2$ in varying concentrations ranging from 1.5 mM to 6 mM, 0.2 mM dNTPs (InVitrogen), probe (0.1 μM) and primer pairs (0.8 μM each) (Proligo, Paris, France). Primer and probes were designed as follows, Fw $\beta-$actin: 5′ AGGTGCGTGATGGTGGG-CATG, Rv $\beta-$actin: 5′ TCCATGTCGTCCCAGTT GGTGA, Pr $\beta-$actin: 5′ CGACGAGGCCCA-CAGCAAGAGAGGCATCCT (54°C). TGFβ(54°C) and IL10 (53°C) designed according to Ref. [18], the IL10 probe was modified to: 5′ GGCTACGGCG CTGTCATCGATTTTC. All probes were modified with 6-FAM and TAMRA on the 5′ and 3′ end, respectively. In order to correct for the differing amounts of cDNA content between the samples, all copy numbers were multiplied by a correction factor. This factor was calculated for each sample by dividing the highest β-actin copy number with the β-actin copy number of the sample.

T-cell stimulations

The cell line KV1919 was rendered apoptotic by irradiation (60Gy) and cocultured with immature DCs for 24 h. The DCs were then transduced with AdLacZ or AdCD40L or left untransduced. At day 1 post-transduction, DCs and autologous T-cells where cocultured at a 1:10 ratio for 3 days whereupon 80 U/ml IL2 was added. After two additional days of culture, the T-cells were harvested and stimulated with KV1919 cells at a 1:4 ratio for 2 h and stained for intracellular IFNγ according to the manufacturer's protocol (BD Biosciences, San Diego, CA).

Results

CD40L- engineering matures DCs and downmodulates IL10 and TGFβ receptors

DCs were transduced with adenoviral vector transferring the cDNA for CD40L. The transgene could be detected during the life span of the DCs (Fig. 1a–c). After 7 days of culture, viability was lost (20%) and the experiments were terminated. At day 3 post-transduction, DCs were analyzed for expression of the maturation marker CD83 (Fig. 1d–f). AdCD40L transduced DCs showed high CD83 expression while untransduced DCs or DCs transduced with control vector containing LacZ had modest expression. Instead, the immature DCs expressed the IL10 receptor while CD40L-engineered DCs did not (Fig. 1g–i). The DCs where further analyzed for the expression of the TGFβ receptors I, II and III. The results from three separate experiments are summarized in Table 1. As expected, only TGFβ receptor I was detectable on DCs since this is the only TGFβ receptor normally found on cells of the hematopoetic lineage (Fig. 2). Interestingly, TGFβ receptor I expression diminishes upon CD40L gene transfer.

AdCD40L-engineered DCs circumvent IL12 exhaustion

Immature DCs from different donors were differentiated from monocytes using IL4 and GM-CSF. At day 6, the immature DCs were genetically engineered with CD40L. The total volume of medium was collected for cytokine analysis at days 2, 3 and 7 in order to measure freshly secreted IL12 at different time points. As a positive control of activation, immature DCs were cultured with TNFα,which was added after each medium change to give a continuous stimulus similar to the continuous stimulation by the transferred CD40L gene. After day 7, viability was lost and the experiment reached a natural end point. Genetically engineered DCs showed high IL12 production, which started early and continued for a week (Fig. 3a) (day 3 not shown). TNFα-activated DCs also produced IL12 (day 3 shown), but at a lower rate compared to AdCD40L-DCs ($\sim$7,000 pg/ml vs > 50,000 pg/ml) (Fig. 3e). In the Figure, IL12 was measured by ELISA that did not detect biologically active IL12. Therefore, the experiments were later repeated and screened with CBA instead. This method detected the biologically activated heterodimeric form of IL12 and confirmed the ELISA data (data not shown). The addition of recombinant IL10 into the cultures, in an attempt to mimic the IL10-rich tumor environment, did not considerably alter IL12 expression in the AdCD40L-DC cultures. On the other hand, the TNFα-matured DCs were strongly affected by IL10 addition which completely abrogated IL12 production in two of three donors (Fig. 3e). The supernatants were further evaluated for the presence of other Th1/Th2- and

Fig. 1 Human genetically engineered DCs were analyzed for expression of the transgene CD40L (**a**: day 1, **b**: day 3 and **c**: day 5) the maturation marker CD83 (**d**: untransduced, **e**: AdLacZ, **f**: AdCD40L), and the IL10 receptor (IL10R; **g**: untransduced, **h**: AdLacZ and **i**: AdCD40L) by flow cytometry. The experiments were repeated at least three times with similar results
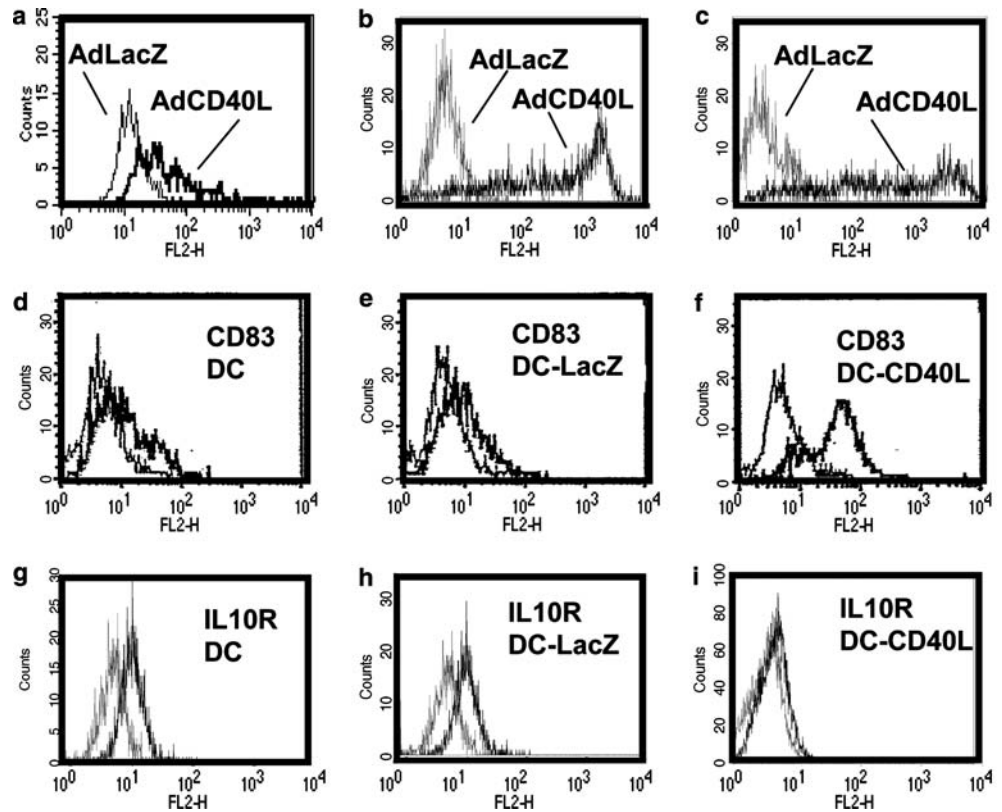
inflammatory cytokines. TNFα was initially upregulated by the genetically engineered DCs but was then swiftly downregulated (Fig. 3b). In this case, addition of recombinant IL10 substantially decreased TNFα release. IFNγ was detected at day 2, but the highest expression was found at day 7 (Fig. 3c). The inhibitor IL10 had no effect on IFNγ production. TNFα-stimulated DCs did not express IFNγ (data not shown),which further argues the use of AdCD40L-modified DCs. Interestingly, IL10 was upregulated early by maturing CD40L-modified DCs but was undetectable by days 3 (data not shown) and 7 (Fig. 3d). Since control vector (AdLacZ)-modified DCs did not express any of the cytokines, we conclude that the expression patterns are due to the CD40L gene

and not due to the viral vector. However, the inflammatory response cytokine IL8 was upregulated in response to both the control vector and CD40L-vector, indicating that IL8 may be triggered by the adenoviral backbone proteins themselves (Table 1). IL6 was also produced in response to control- transduced DCs although CD40L-engineered DCs induced an even higher expression of IL6 (Table 1). As for IL1β, CD40L-transduced DCs induced levels only slightly higher than LacZ or untransduced DCs. CBA analysis of IL2, IL4 and IL5 showed undetectable levels of those cytokines, suggesting that there was no contamination by lymphocyte populations (which are the main producers of these cytokines).

Table 1 DCs untransduced, transduced with control vector Ad-LacZ or AdCD40L were screened for TGFβRs (immunocytochemistry) and inflammatory cytokines (cytometric bead array)

Molecule	Untransduced	AdLacZ	AdCD40L
TGFβR-I	+ + +	+ + +	+
TGFβR-II	−	−	−
TGFβR-III	−	−	−
IL8	3,998 pg/ml	21,200 pg/ml	18,071 pg/ml
IL1β	20 pg/ml	21 pg/ml	86 pg/ml
IL6	41 pg/ml	73 pg/ml	768 pg/ml

(−) negative staining, (+) a few 1–2% detectable cells and (+ + +)expression by >70% of cells compared to control staining with irrelevant antibodies. The cytokine concentrations are from one donor. The experiment was repeated twice with similar results.

Genetically modified DCs produce IL12 in the presence of tumor cell lines

Genetically engineered DCs were protected from IL12 exhaustion even in the presence of tumor cells. Immature DCs from three different donors were transduced and mixed with the four bladder cancer cell lines. At day 3, supernatants were harvested and analyzed for cytokine expression. Upon CD40L-transduction and tumor cell coculture, DCs produced high amounts of IL12 with the exception of DCs cocultured with VMCUB-1 (Fig. 4a). IFNγ was produced at similar levels in DC/tumor cell/CD40L cocultures as seen in DC cultures without tumor cells. However, IFNγ was not

Fig. 2 DCs were analyzed by cytochemistry for expression of TGFβ receptors I, II and III day 3 post-transduction. In this figure, DCs from one donor is exemplified. The experiment was repeated with three different donors and summarized in Table 1
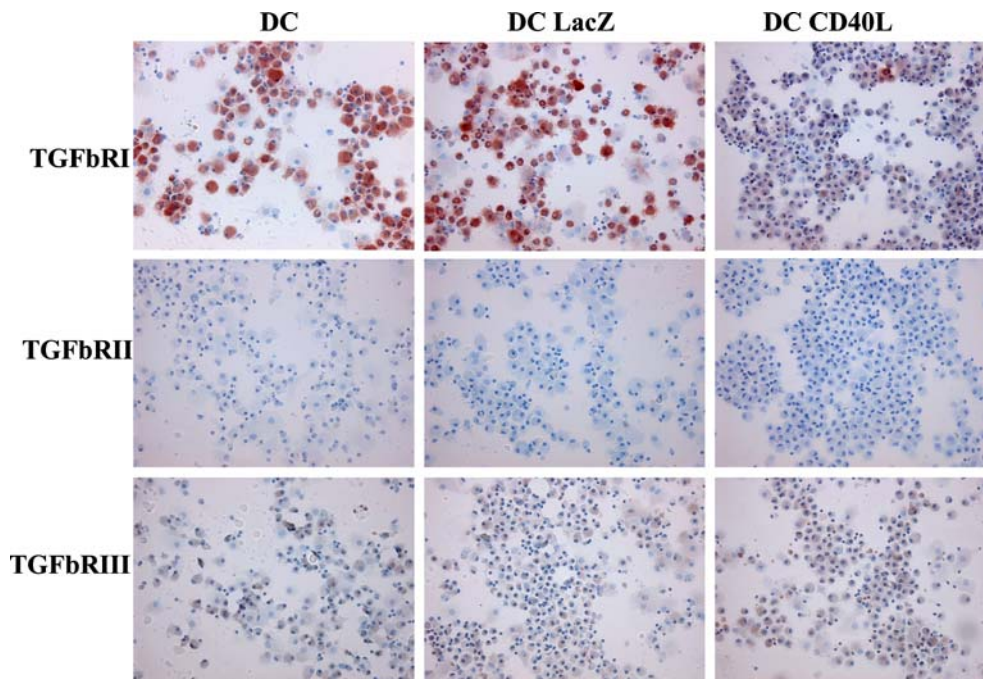
upregulated in coculture with the cell line VM-CUB-1 (Fig. 4b). VM-CUB-1, as well as T24 and KV-19-19, had a negative effect on TNFα production (Fig. 4c). CD40L-stimulated DCs produced TNFα in the range of 3,500 pg/ml. When the DCs were cocultured with the tumor cell lines, the production decreased to less than 1,000 pg/ml. With the exception of RT112, coculture of DCs and tumor cells suppressed the initial IL10 production seen upon CD40L activation of DCs cultured alone (compare Fig. 3d and 4d).

IL10 and TGFβ may suppress but not abolish Th1 responses by genetically engineered DCs

The bladder carcinoma tumor cell lines derived were analyzed for expression of IL10 and TGFβ. None of the cell lines expressed IL10 as determined by cytometric bead array and quantitative PCR (data not shown) but all lines expressed similar levels of TGFβ (data not shown). Although the cell lines were negative for IL10, this cytokine is often present in freshly isolated bladder tumor tissue (Loskog et al., in preparation). Furthermore, IL10 may be generated in vivo by other cell types surrounding the growing tumor including regulatory T-cells. Therefore, recombinant IL10 was added to the DC−tumor cell cocultures to create a more realistic tumor milieu. Figure 5 demonstrates that recombinant IL10 may regulate IL12 production by DCs. Still, even with IL10 added, all the cocultures had IL12 production at similar or higher levels than that of TNFα-matured DCs (compare Figs. 5 and 3e). The same was seen for IFNγ and TNFα release (Fig. 6). The expression of these cytokines were similar to DCs cultured without tumor cells or IL10. In Figs. 5 and 6, the data exemplify that the various cell lines seemed to have different regulatory effects on DCs since the cytokine levels vary depending on the tumor cell line in the culture. In particular, VM-CUB-1 partly downregulated the Th1 responses mounted by genetically engineered DCs.

Genetically engineered DCs stimulate T cells to produce IFNγ

To further strengthen the hypothesis that AdCD40L-transduced DCs are potent stimulators of anti-tumor responses, DCs pulsed with apoptotic cells from the tumor cell line KV1919 were incubated with autologous T-cells for 5 days. After incubation with DCs, the T-cells were stimulated with KV1919 cells for 2 h and then stained for intracellular IFNγ and analyzed by flow cytometry. T-cells stimulated with untransduced or control-transduced DCs showed background IFNγ production probably due to alloreaction to the cell line. However, T-cells stimulated with DCs transduced with AdCD40L showed increased levels of IFNγ production (Fig. 7). Since KV1919 does not express MHC class II, the 2-h stimulation of T-cells prior to IFNγ staining did not affect the CD4 cell population.

Discussion

In order to develop effective immunotherapies for the treatment of cancer, we need to understand and learn how to control the underlying mechanisms in the tumor milieu that dictate local immune responses. DC

Fig. 3 Supernatants were screened for cytokine production by CD40L-modified DCs +/− rIL10 using ELISA (**a**: IL12) and CBA (**b**: TNF, **c**: IFNγ and **d**: IL10). The total volume of supernatants from three different donors was collected at three time points: days 2, 3 and 7 (day 3 not shown). IL12 expression from TNFα-matured DCs (day 2) from three donors are shown in Fig. 2e. *None* = native unstimulated DCs. Since IL12 concentrations were much higher for the CD40L groups the CD40L bars were cut-off in order to visualize the other groups. The highest standard was 2,000 pg/ml but even with high dilutions some values went beyond this limit. *Error bars* represent SEM values. The cytokine levels in the AdCD40L +/− IL10 groups were significantly different from the control groups ($\alpha = 0.01$, $P < 0.01$, independent t-test)
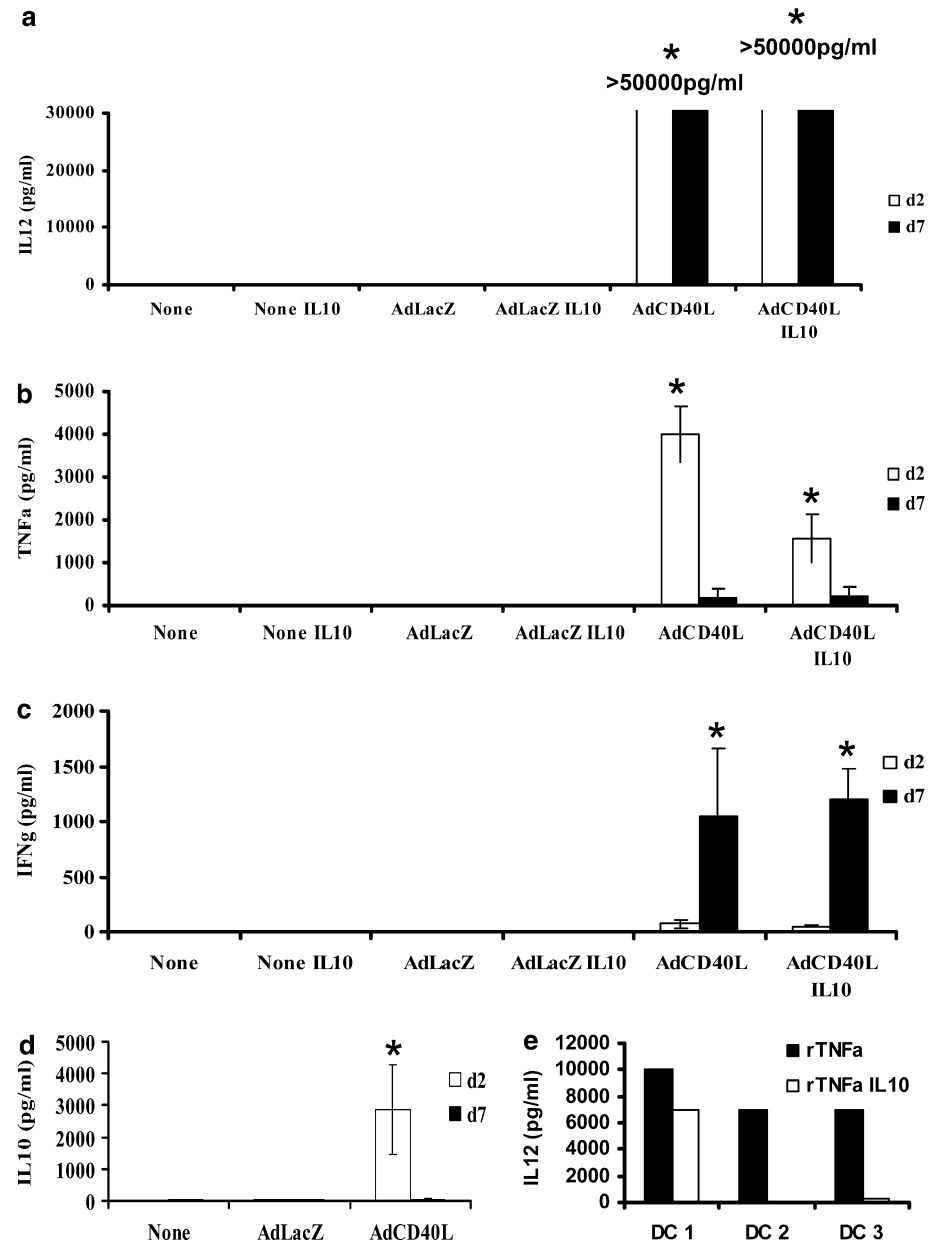
maturation links innate responses to adaptive immunity. Fujii et al. demonstrated that CD40L is required for this linkage to occur since CD40$^{-/-}$ mice could not mount adaptive responses [19]. CD40L is known for its capacity to mature DCs and initiate Th1-type responses against tumors [20, 21]. However, DCs matured with TNFα are used in standard protocols for DC vaccine development today. In the present study, DCs genetically engineered to express CD40L were evaluated for their capacity to mature and maintain Th1-related cytokine production. The adenoviral vector system was selected since it does not integrate into the host genome. This means that transgene expression is transient, which is appropriate since the lifespan of DCs is limited. Adenoviral vectors have been utilized for in vivo gene transfer and by localized injection sites, and has been proven safe and feasible [22, 23].

In this study, we investigated the cytokine production by genetically engineered DCs during their lifespan of approximately 7 days in order to anticipate what effect they may have in vivo. It is important to note that the DCs will be Th1-promoting without shifting the cytokine profile during the vaccinations of cancer patients. Immature DCs were easily transduced with adenoviral vectors without apparent toxicity. Our results showed that DCs modified to express CD40L continuously produced CD40L during their life span. The stimulation of CD40 on DCs leads to IL12 production. In our cultures, we could detect IL12 production during 7 days of culture with total medium changes at several time points. After
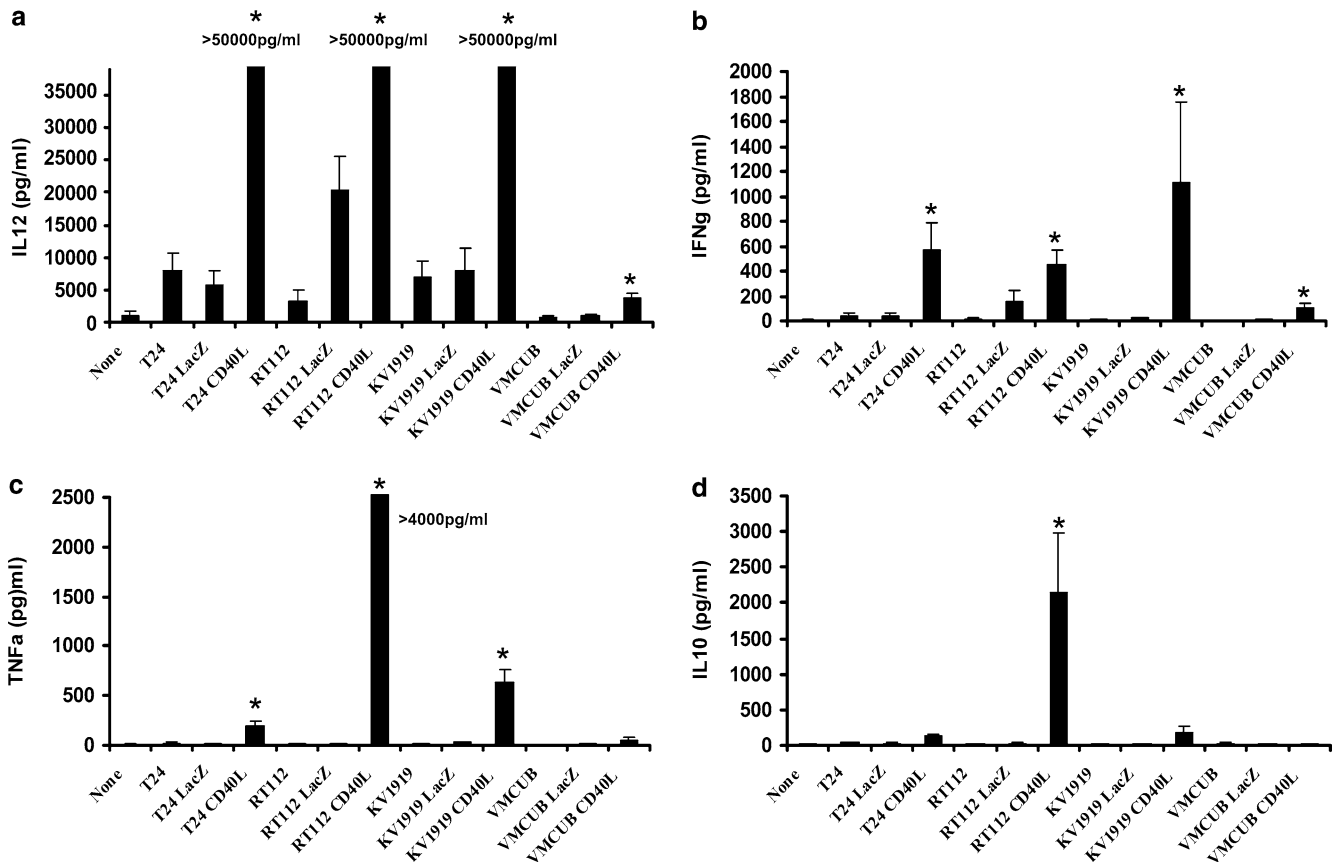
Fig. 4 Engineered DCs from four different donors were incubated with no stimuli or with tumor cell lines. Supernatants were harvested at day 3. Cytokine expression was detected using ELISA (**a**: IL12) and CBA (**b**: IFNγ, **c**: TNFα and **d**: IL10). *None* = native unstimulated DCs. *Error bars* represent SEM values. The IL12 and IFNγ concentrations of CD40L groups were significantly different from control groups (all cell lines: $\alpha = 0.01$, $P < 0.01$, independent t-test). The TNFα concentration was significantly different in the CD40L groups for T24, RT112 and KV1919 ($\alpha = 0.01$, $P < 0.01$, independent t-test). IL10 levels were significantly different in the CD40L group compared to control groups only in experiments performed with RT112 ($\alpha = 0.01$, $P < 0.01$, independent t-test)

7 days of culture, the viability of the DCs decreased but IL12 was still detected at high levels indicating that the few remaining viable cells were still functional. Also, TNFα and IFNγ could be detected in the supernatants of CD40L-expressing DCs. Vieira et al. demonstrated that addition of IFNγ was essential for increased IL12 production by DCs upon in vitro maturation [24]. In the present paper, the IFNγ production by CD40L-matured DCs may be an explanation for the high IL12 induction compared to TNFα-matured DCs which lacked IFNγ. Addition of IL10 into the DC cultures did not prevent IL12 or IFNγ production. These results can, in part, be explained by the downmodulation of IL10 receptor upon CD40L stimulation which presumably renders the DCs resistant to the regulatory effects of IL10. However, DCs cocultured with tumor cell lines were less resilient when IL10 was added. The supernatants from DC/tumor/CD40L cocultures contained high levels of IL12 and

IFNγ, with the exception of the tumor cell line VM-CUB-1 that seemed to have a negative effect on the DCs in general. When IL10 was added, DCs produced less IL12 but levels were still comparable to those with TNF-α maturation. None of the cell lines produced IL10 themselves, but they did produce substantial levels of TGFβ mRNA. Even so, they had differential effects on DCs from the same donor. Further, the receptor for TGFβ (TGFβR-I) was suppressed, but not lacking, on CD40L-modified DCs. Finally, we cannot exclude the presence of other immune inhibitory molecules, particularly in the case of VM-CUB-1.

Similar results can be obtained if tumor cells instead of DCs are transduced with AdCD40L indicating that the continuous CD40L-stimulation per se is more important than the genetic modification. Even if part of the DCs are transduced, CD40L-expressing cells would interact with a neighboring DC and induce maturation and IL12 production (our own unpublished data). Control vector (AdLacZ) transduction of human immature DCs had no effect on DC maturation in our study. However, Schumacher et al. demonstrated that DCs could mature upon transduction with adenovirus. The maturation level was characterized as being higher than using TNFα but lower than using CD40L, and only a subset of transduced cells produced IL12 [25]. In the present study, the control vector induced production of the inflammatory cytokines IL6 and particularly IL8, although this was not enough to drive DC maturation.

Fig. 5 IL12 production was evaluated at day 3 for engineered DCs cocultured tumor cells +/− addition of recombinant IL10. Note that VM-CUB-1 stimulated DCs have lower expression of IL12 than in the other groups. Cytokine levels were measured by ELISA. *Error bars* represent SEM values. The experiment was repeated with three different donors
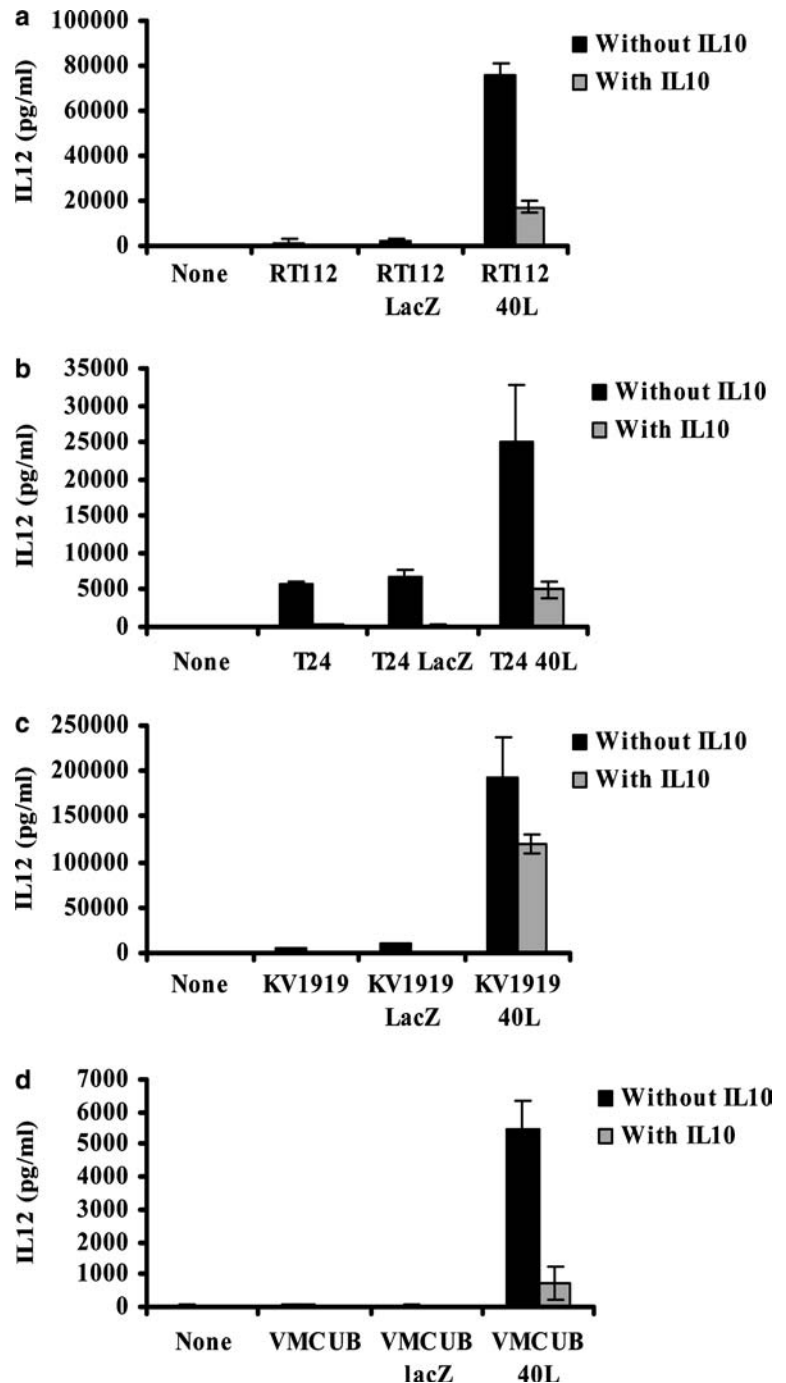
Kikuchi et al. showed that in murine models, CD40L-engineered DCs could elicit tumor-specific CD8 T-cell responses [26]. In another study, they demonstrated that AdCD40L vector could be administered to the tumor area followed by injection of immature DCs at the tumor site [27]. This combination also mounted tumor-specific CD8 T-cell responses. Liu et al. used CD40L-transduced murine DCs to vaccinate mice and subsequently challenged them with lung cancer and lymphoma cell lines. The vaccinated mice were completely resistant to tumor outgrowth [28]. In previous work, we had used AdCD40L vectors for direct injection into MB49 bladder cancer nodules. Apart from monitoring tumor growth and T- cell activation, we studied the cytokine deviation at the tumor site as well as in lymph nodes [17]. The MB49 tumor milieu was rich in IL10 and TGFβ, and both cytokines were suppressed upon treatment with AdCD40L. Instead, high levels of IL12 were detected. Murine DCs have also been modified to express TNFα, IL12, IL18, Flt3 ligand, and IL4 to gain enhanced Th1-type responses in various models [29–33].

The expression of IFNγ (**a**) and TNFα (**b**) was affected by the addition of recombinant IL10 into DC-tumor cocultures at day 3. However, Th1 dominance could not be completely abolished. The cytokine levels were measured by cytometric bead array. The experiment was repeated with three different donors
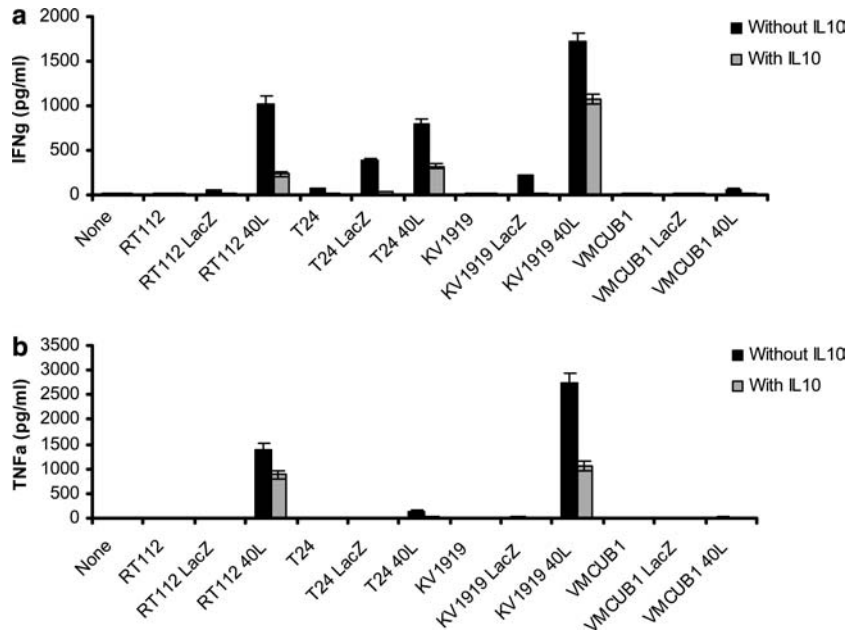

In this study, we demonstrate that CD40L-modified DCs can induce IFNγ producing T -cells. Unfortunately, freshly- isolated bladder cancer tumor cells show low viability in vitro. Therefore, we used an allogeneic system utilizing the cell line KV1919 as a tumor source. T -cells cocultured with CD40L-expressing DCs produced higher amounts of IFNγ when correcting for a low background alloresponse. So far, genetically modified DCs have only been evaluated for their maturation stage and capacity to stimulate T- cell responses. In the present study, the impact of the hostile tumor milieu (Tr1/Th3-type cytokines) was under consideration. We argue that AdCD40L-

engineered DCs continuously produce IL12 and are resistant to immune escape mechanisms such as IL10 and TGFβ in the tumor environment. Therefore, CD40L-engineered DCs may circumvent many of the obstacles hampering immunotherapy by creating a milieu for effective Th1-mediated tumor eradication.

It is a challenge to develop potent immunotherapies that overcome the immune escape barriers induced by tumor cells [34]. We demonstrate here that engineering DCs to express CD40L renders them resistant to immune deviation by IL10, and also renders them capable of producing IL12 continuously. In this study, we also studied the activation of DCs in an immunosuppressive tumor milieu. AdCD40L-modified DCs cocultured with tumor cell lines expressing TGFβ still produced high amounts of IL12. In conclusion, our results argue for the application of CD40L-based immunogene therapy of IL10/TGFβ-associated tumors such as bladder carcinoma. Further, these results demonstrate that CD40L is

Fig. 7 T-cells stimulated with DCs (loaded with apoptotic KV1919) for 5 days were incubated with viable KV1919 cells for 2 h. The T-cells were then analyzed by flow cytometry for intracellular IFNγ (FITC). The T-cells were gated by CD3(-APC) and then visualized as CD8 (PE)-positive T cells on the y-axis. The percentage of IFNγ-positive cells within the CD8 positive cell population is shown in the figure
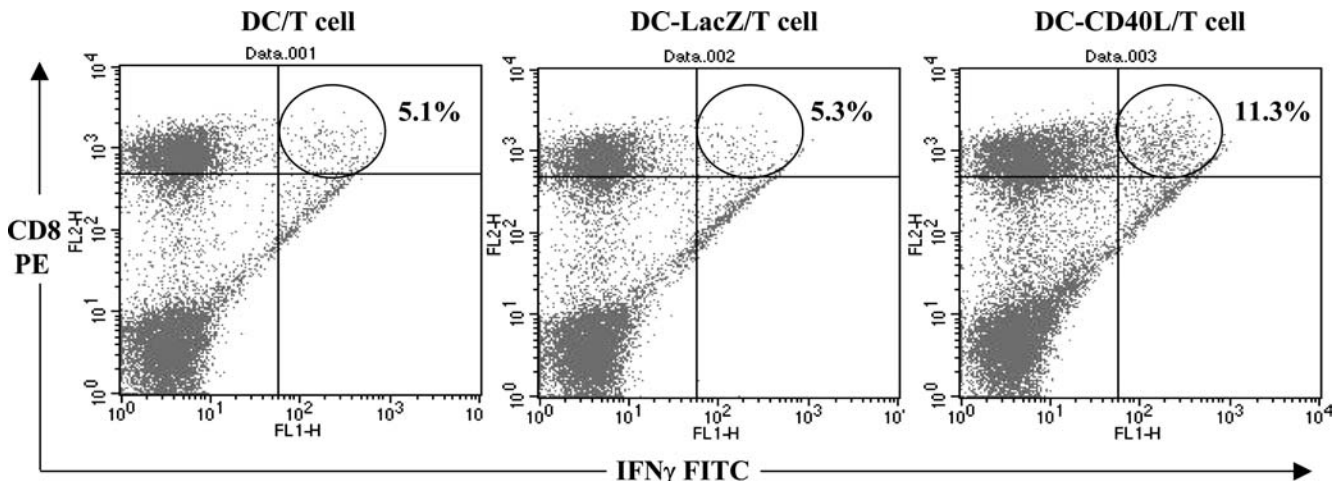
superior to TNFα in enhancing IL12 and IFNγ production and should be considered for DC maturation in DC vaccination strategies.

References

1. Swain SL (1999) Helper T cell differentiation. Curr Opin Immunol 11:180
2. Sogn JA (1998) Tumor immunology: the glass is half full. Immunity 9:757
3. Villunger A, Strasser A (1999) The great escape: is immune evasion required for tumor progression? Nat Med 5:874
4. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000) Immunobiology of dendritic cells. Annu Rev Immunol 18:767
5. Lanzavecchia A, Sallusto F (2001) Regulation of T cell immunity by dendritic cells. Cell 106:263
6. Lanzavecchia A (1998) Licence to kill. Nature 393:413
7. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10:942
8. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH (2001) Regulatory CD4 + CD25 + T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 61:4766
9. Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH (2002) Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. J Immunol 168:4272
10. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 169:2756
11. D'Orazio TJ, and Niederkorn JY (1998) A novel role for TGF-β and IL-10 in the induction of immune privilege. J Immunol 160:2089
12. Gilboa E (1999) How tumors escape immune destruction and what we can do about it. Cancer Immunol Immunother 48:382
13. Seo N, Hayakawa S, Takigawa M, Tokura Y (2001) Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4 + T-regulatory cells and systemic collapse of antitumour immunity. Immunology 103:449
14. Chang GC, Lan HC, Juang SH, Wu YC, Lee HC, Hung YM, Yang HY, Whang-Peng J, Liu KJ (2005) A pilot clinical trial of vaccination with dendritic cells pulsed with autologous tumor cells derived from malignant pleural effusion in patients with late-stage lung carcinoma. Cancer 103:763
15. Reichardt VL, Brossart P (2005) Dendritic cells in clinical trials for multiple myeloma. Methods Mol Med 109:127
16. Brody JD, Engleman EG (2004) DC-based cancer vaccines: lessons from clinical trials. Cytotherapy 6:122
17. Loskog A, Dzojic H, Vikman S, Ninalga C, Essand M, Korsgren O, Totterman TH (2004) Adenovirus CD40 ligand gene therapy counteracts immune escape mechanisms in the tumor Microenvironment. J Immunol 172:7200
18. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C (2001) An overview of real-time PCR: applications to quantify cytokine gene expression. Methods 25:386
19. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM (2004) The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med 199:1607
20. Kuwashima N, Kageyama S, Eto Y, Urashima M (2001) CD40 ligand immunotherapy in cancer: an efficient approach. Leuk Lymphoma 42:1367
21. Tong AW, MJ Stone (2003) Prospects for CD40-directed experimental therapy of human cancer. Cancer Gene Ther 10:1
22. Wen SF, Mahavni V, Quijano E, Shinoda J, Grace M, Musco-Hobkinson ML, Yang TY, Chen Y, Runnenbaum I, Horowitz J, Maneval D, Hutchins B, Buller R (2003) Assessment of p53 gene transfer and biological activities in a clinical study of adenovirus-p53 gene therapy for recurrent ovarian cancer. Cancer Gene Ther 10:224
23. Kuball J, Wen SF, Leissner J, Atkins D, Meinhardt P, Quijano E, Engler H, Hutchins B, Maneval DC, Grace MJ, Fritz MA, Storkel S, Thuroff JW, Huber C, Schuler M (2002) Successful adenovirus-mediated wild-type p53 gene transfer in patients with bladder cancer by intravesical vector instillation. J Clin Oncol 20:957
24. Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kalinski P (2000) Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J Immunol 164:4507
25. Schumacher L, Ribas A, Dissette VB, McBride WH, Mukherji B, Economou JS, Butterfield LH (2004) Human dendritic cells maturation by adenovirus transduction enhances tumor antigen-specific T-cell responses. J Immunother 27:191
26. Kikuchi T., Moore MA, Crystal RG (2000) Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. Blood 96:91
27. Kikuchi T, Miyazawa N, Moore MA, Crystal RG (2000) Tumor regression induced by intratumor administration of adenovirus vector expressing CD40 ligand and naïve dendritic cells. Cancer Res 60:6391
28. Liu Y, Zhang X, Zhang W, Chen Z, Chan T, Ali K, Jia Z, Xiang J (2002) Adenovirus-mediated CD40 ligand gene-engineered dendritic cells elicit enhanced CD8 + cytotoxic T-cell activation and antitumor immunity. Cancer Gene Ther 9:202
29. Zhang W, Chen Z, Li F, Kamencic H, Juurlink B, Gordon JR, Xiang J (2003) Tumour necrosis factor-alpha (TNF-alpha) transgene-expressing dendritic cells (DCs) undergo augmented cellular maturation and induce more robust T-cell activation and anti-tumour immunity than DCs generated in recombinant TNF-alpha. Immunology 108:177
30. Kuipers H, Heirman C, Hijdra D, Muskens F, Willart M, van Meirvenne S, Thielemans K, Hoogsteden HC, Lambrecht BN (2004) Dendritic cells retrovirally overexpressing IL-12 induce strong Th1 responses to inhaled antigen in the lung but fail to revert established Th2 sensitization. J Leukoc Biol 76:1028
31. Xia D, Li F, Xiang J (2004) Engineered fusion hybrid vaccine of IL-18 gene-modified tumor cells and dendritic cells induces enhanced antitumor immunity. Cancer Biother Radiopharm 19:322
32. Liu Y, Huang H, Chen Z, Zong L, Xiang J (2003) Dendritic cells engineered to express the Flt3 ligand stimulate type I immune response, and induce enhanced cytotoxic T and natural killer cell cytotoxicities and antitumor immunity. J Gene Med 5:668
33. Kaneko K, Wang Z, Kim SH, Morelli AE, Robbins PD, Thomson AW (2003) Dendritic cells genetically engineered to express IL-4 exhibit enhanced IL-12p70 production in response to CD40 ligation and accelerate organ allograft rejection. Gene Ther 10:143
34. Igney FH, Krammer PH (2002) Immune escape of tumors: apoptosis resistance and tumor counterattack. J Leukoc Biol 71:907