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Generation of potent anti-tumor immunity in mice by interleukin-12-secreting dendritic cells

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Abstract To induce cytolytic immunity, dendritic cells (DCs) need to release bioactive interleukin-12 (IL-12) p70 heterodimeric molecules. To study the role of IL-12 for the generation of an anti-tumor immune response, we generated two classes of DCs. (1) DCs were initiated to secrete IL-12 by exposure to LPS/IFN- γ for 2 h resulting, as demonstrated in vitro, in continued IL-12 release for another 24 h (termed active DCs). (2) DCs were exposed to LPS/IFN- γ for 24 h and injected into mice at a time point when IL-12 production had ceased (termed exhausted DCs). These two classes of DCs were probed for their capacity to induce a cytolytic anti-tumor immune response in vivo in a syngeneic mouse tumor model. The mouse tumor cell line K-Balb was engineered to express neomycin phosphotransferase (NPT) as a model tumor antigen. DCs were charged with various NPT-derived antigens, including recombinant NPT protein, whole tumor cell lysate and NPT-derived synthetic peptides, and the induction of in vivo anti-tumor immunity was determined by measuring tumor growth. Only the injection of active DCs, i.e., cells that maintained the capacity to secrete IL-12, but not exhausted DCs that had lost the ability to produce IL-12, resulted in a measurable deceleration of growth of K-Balb-NPT tumors. This anti-tumor immune response was most pronounced when using recombinant protein as an antigen source, which was evident in a prophylactic as well as in a therapeutic setting. The absence of a response to parental K-Balb tumors confirmed the

antigen specificity of the anti-tumor immune response. Together these data provide evidence for the unique capacity of actively IL-12 secreting DCs to trigger effective anti-tumor immunity using exogenous tumor antigens.

Keywords Antigen presenting cell (APC) · Dendritic cell (DC) · Interleukin-12 (IL-12) · Cytotoxic T lymphocyte (CTL) · Cytolytic anti-tumor immune response

Introduction

Dendritic cells (DCs) are a sparsely distributed, migratory group of bone marrow-derived leukocytes that are specialized for the uptake, transport, processing and presentation of antigens to T cells [14, 18]. The balance between the induction of T helper (Th) cells and cytotoxic T lymphocytes (CTLs) and the subsequent polarization of the immune response is determined by the context in which antigen is presented. Exogenous antigens are phagocytosed, which introduces them into the MHC class II pathway where they are presented to CD4⁺ Th cells. T cell receptors on CD8⁺ CTLs recognize endogenous antigens bound to MHC class I molecules. Since CTLs are able to lyse tumor cells directly and eradicate large tumor masses in vivo [33], these cells have received the most attention in anti-tumor immune therapy. Hence, the polarization of the immune response towards CTL activation is of major interest for the design of new anti-tumor immune therapies. DCs have emerged as the most potent adjuvants for cancer immunotherapy [13, 28]. Tumor-derived antigens are presumably processed and presented on MHC class II molecules at the DC cell surface and thus, as they represent cellular proteins, are likely to trigger a polyclonal expansion of Th cells. The generation of a CTL response would require that these exogenous proteins have to “cross-over” to the endogenous pathway to gain access to the MHC class I pathway [3].

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Recent reports have demonstrated that interleukin (IL)-12 secreted from DCs is a mandatory requirement for the polarization of a type 1 Th cell (Th1) response [24] that facilitates cytolytic immunity. This finding was corroborated by other investigators, who found that immunization with tumor-lysate pulsed DCs in cancer-bearing hosts was exclusively effective in conjunction with IL-12 administration [32]. Subsequent reports revealed that DCs must reach a certain state of maturation before they acquire the capability to secrete IL-12. This was amended by the finding that IL-12 release from DCs is highly dependent on the type of the maturation stimulus and the cytokine milieu during stimulation [16]. In addition, it is known that the ability for IL-12 release is restricted to DCs early after induction of maturation and is switched off at later time points [17]. Thus, the capacity of DCs to efficiently prime Th1 cells in order to mediate tumoricidal CTL activity appears to depend (1) on the stimulus itself and (2) on the time kinetics after exposure to an appropriate maturation stimulus to assure that the DCs have not exhausted their capacity to secrete IL-12. The purpose of this work was to identify strategies for the generation of DCs that are active in IL-12 release and are therefore per se enabled for the induction of cytolytic anti-tumor immunity.

Materials and methods

Mice

Balb/c mice were bred in the animal facility of the Institute of Immunology (Medical University of Vienna, Austria). All mice were maintained under specific pathogen-free conditions following a protocol approved by the Institutional Animal Care and Use Committee and in accordance with the ethical guidelines of the Federal Ministry of Science and Culture. Experiments were conducted with 6–8-week-old female mice, and each treatment group comprised five mice, which were finally killed according to properly established protocols [7] and government regulations.

Cell culture medium

The dendritic cell culture medium used (subsequently termed complete medium, CM) was RPMI 1640 with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 10 mM Hepes, 1 mM sodium pyruvate, 0.1 mM nonessential aminoacids and 50 µM 2-mercaptoethanol (all from LifeTechnologies, Grand Island, N.Y.). CM was supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, PAA, Linz, Austria).

Generation of DCs

Dendritic cells were generated from mouse bone marrow according to an established protocol [10] with minor

modifications. Briefly, long bones of female Balb/c mice were dislodged and remaining tissue and muscles were removed with sterile gauze. The bones were sterilized in 70% (v/v) EtOH for 5 min, air-dried and put into sterile phosphate-buffered saline (PBS, GIBCO Industries, Inc., Tulsa, Okla.). The ends of the bones were cut with sterile scissors, and bone marrow was flushed with sterile PBS from femurs and tibias. Red blood cells were lysed in a hypotonic buffer for 10 min at 4°C. The bone marrow was washed, and cells were re-suspended in CM supplemented with 10% (v/v) FCS. After counting on a Coulter Particle and Size Analyzer (Coulter Electronics Ltd., UK), the cells were cultured in 24-well plates (Iwaki, Japan) at a concentration of 1×10^6 per well in CM supplemented with 10% (v/v) FCS in the presence of a previously optimized concentration of 5 ng/ml recombinant murine IL-4 and 3 ng/ml recombinant murine GM-CSF (Pharmingen, San Diego, Calif.). Half of the medium (including all supplements) was replaced every 2nd day. Usually, this process removed non-adherent granulocytes, whereas clusters of developing DCs remained loosely attached. After 6 days, DC recovery was determined using the TruCount system (Pharmingen, San Diego, Calif.). DCs were stained with anti-CD11c mAb. The number of DCs averaged 0.7×10^6 per well (i.e., 70% recovery). Less than 10% of these cells used for in vivo and in vitro experiments were macrophages (as determined by the macrophage marker F4/80).

Tumor cells and tumor antigens

The Kirsten sarcoma virus transformed Balb/3T3 cell line K-Balb (ATCC, Manassas, Va.) was maintained in tissue culture at 37°C in CM supplemented with 10% (v/v) heat-inactivated FCS. The retrovirus-based vector LNL6 expressing the *neomycin phosphotransferase* (NPT) was used to generate K-Balb-NPT cells. Briefly, 10^5 K-Balb wildtype (wt) cells were co-cultivated with 10^5 irradiated (6,000 rad) LNL6 producer cells for 3 days followed by selection for 7 more days with G418 (1 mg/ml, Invitrogen, Carlsbad, Calif.), resulting in stably transduced K-Balb-NPT tumor cells. All tumor cell lines were routinely screened for mycoplasma contamination every 6 months and remained negative (Ridascreen Mycoplasma IFA, R-Biopharm AG, Darmstadt, Germany).

By using various antigen preparations, the immunoreactivity to three different antigen sources could be tested. From K-Balb as well as K-Balb-NPT cell lines, tumor cell lysates were generated as a source of TAAs. Tumor cells were washed, reconstituted at a cell density of 5×10^7 in 1 ml pure water and lysed by repeated freeze-thaw cycles. The lysates were centrifuged at 500 g for 7 min to remove insoluble cell fragments, and the supernatant was adjusted to isotonic concentration with 10× PBS. The protein concentration of cell lysates was determined with a Protein Micro Assay (BioRad

Laboratories, Hercules, Calif.), and lysates were stored in 100 μ l aliquots at -80°C . For the use of peptides as TAA, the NPT-derived nonamers *GYDWAQQTI* and *PVLFVKTDL*, as predicted to be H-2 K^d restricted by the SYFPEITHI database (<http://www.syfpeithi.de>), were synthesized and purified by HPLC (VBC Genomics, Vienna, Austria).

Recombinant NPT protein was generated using high level expression and purification of 6x His-tagged proteins via Ni-NTA technology (Qiagen, Chatsworth, Calif.). Briefly, the NPT gene was amplified by PCR using the pLXSN vector (gene bank accession number MA28248) as template. The primer sequences used for the amplification reaction were 5' *CGG GAT CCG GAT TGC ACG CAG GTT CT* 3' and 5' *GCA AGC TTT CAA GAA GGC GAT AGA AGG CG* 3'. The PCR mix was purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, Calif.), analyzed on a 0.8% (w/v) agarose gel, and purified from the gel with a Concert Rapid Gel Extraction Kit (Invitrogen, Carlsbad, Calif.). The PCR fragment was digested with BamHI and HindIII and ligated into the pQE30 6x His-tag-construct. Competent XL-1 blue *Escherichia coli* cells were prepared as previously described [26]. To obtain bacterially expressed 6x His-tagged protein, NPT was induced with 1 mM IPTG at an $\text{OD}_{600} = 0.6\text{--}0.8$ for 3 h at 37°C . Bacterial cell lysates were loaded onto Ni-NTA spin columns, and 6x His-tagged NPT protein was eluted. The NPT protein was analyzed on a 12.5% (w/v) SDS-polyacrylamid gel (SDS-PAGE). The protein concentration was determined in a spectrophotometer at OD_{595} in comparison to a linear BSA standard. NPT protein was stored at -80°C until loading onto DCs. Endotoxin contamination of recombinant NPT was determined using the Limulus Amebocyte Lysate (LAL) assay (Biowhittaker, Walkersville, Md.) according to the manufacturer's instructions. Polymyxin B sulfate (50 $\mu\text{g}/\text{ml}$; Sigma Aldrich, St. Louis, Mo.) was added to recombinant NPT protein before loading onto DCs to neutralize NPT-contaminating endotoxin, thus preventing DCs from an untimely maturation [4].

To test for the endocytotic and phagocytotic capacity of immature DCs and in order to define the optimal antigen dose, we used FITC-labeled Ovalbumin (FITC-OVA). Immature DCs were exposed to 1 μg OVA-FITC; the efficiency of engulfment was evaluated by flow cytometry and estimated by FITC-positive cells with high mean fluorescence intensity (data not shown). The most effective uptake was achieved with 1 $\mu\text{g}/\text{ml}$ protein, which was not exceeded at higher antigen concentrations (up to 10 $\mu\text{g}/\text{ml}$). For pulsing DCs with TAA on day 6, cells at a concentration of $10^6/\text{ml}$ were exposed to 1 μg recombinant NPT protein, NPT-derived synthetic peptides or K-Balb-NPT-derived whole tumor cell lysate for 2 h at 37°C in CM without serum supplements. Subsequently, the medium was replaced by CM/10% (v/v) FCS.

Maturation of DCs

After antigen loading, DC maturation was induced by incubation with various reagents for 24 h. Immature DCs were exposed to 100 ng/ml LPS from *E. coli* O55:B55 (Sigma, St. Louis, Mo.) alone or in combination with 10 ng/ml IFN- γ (specific activity: $0.2\text{--}1 \times 10^8$ U/mg, Pharmingen, San Diego, Calif.). Alternatively, 20 ng/ml lipoteichoic acid from *B. subtilis* (Sigma, St. Louis, Mo.), 2 $\mu\text{g}/\text{ml}$ Poly (I:C) (Sigma Aldrich Chemie, Germany), 100 ng/ml TNF- α (specific activity: $\geq 1 \times 10^7$ U/mg, PeproTech EC LTD, London, UK) or 10^4 U/ml IFN- α (specific activity: 4.8×10^7 U/mg interferon, VWR International, Vienna) was used for induction of maturation. To deliver a CD40/CD40-ligand (CD40L)-mediated maturation signal, DCs were co-cultured with irradiated (6,000 rad) SJ-NB7 human neuroblastoma cells (kindly provided by T. Look, St. Jude Children's Research Hospital, Memphis, Tenn.) transfected with mCD40L cDNA [8] at a ratio of 2×10^6 DC/ 1×10^6 SJ-NB7mCD40L. Alternatively we used mAbs directed against CD40 that trigger CD40 cross-linking as described previously by Ridge et al. [27]. Briefly, DCs were incubated with IgG2a purified rat anti-mouse CD40 mAb (clone 3/32, Pharmingen, San Diego, Calif.) and overnight with mouse adsorbed goat anti-rat Ig specific polyclonal antibodies (Pharmingen, San Diego, Calif.). The cells were washed after each incubation step to remove excess antibodies with CM/10% (v/v) FCS. For phenotypic characterization, DCs were collected into cold PBS/1% (w/v) BSA/5 mM EDTA, washed and then incubated for 20 min on ice with purified 2.4G2 antibody against FC γ RII (Mouse Fc Block, Pharmingen, San Diego, Calif.) followed by fluorophore-labeled mAb (all from Pharmingen, San Diego, Calif.): FITC-anti-CD11c (clone HL3), FITC-anti-CD40 (clone 3/23), PE-anti-CD80 (clone 16-10 A1), PE-anti-CD86 (clone GL1), PE-anti-MHC II (clone SF1-1.1) and PE-anti-CD11b (clone M1/70). Flow cytometric analysis was performed using a FACScan flow cytometer; data analysis was performed using the CellQuest software (both BD Biosciences, San Jose, Calif.).

Quantification of IL-12 secretion

The amount of bioactive IL-12 p70 was determined in the supernatants of mature DC cultures by ELISA (OptEIA, Pharmingen, San Diego, Calif.). The supernatant of immature DC cultures served as negative control. The cytokine content was calculated using recombinant standards ranging from 4,000 pg/ml to 62.5 pg/ml.

For intracellular cytokine staining $2.5 \times 10^6/\text{ml}$ splenocytes from immunized mice were re-stimulated for 5 days with 2.5×10^5 mitomycin C pre-treated K-Balb-NPT cells (50 $\mu\text{g}/\text{ml}$ for 45 min) in CM/10% (v/v) FCS. On day 0 and 3 of the culture, cells were supplemented with recombinant murine IL-2 (10 ng/ml, Pharmingen,

San Diego, Calif.). A mAb cocktail that included anti-CD3 (clone 145-2C11), anti-CD8 α (clone 53-6.7) and anti-CD4 (clone RM 4-5) was used to determine the T-lymphocyte subset distribution. Prior to cytokine staining (day 5), splenocytes were activated with 50 ng/ml phorbol-myristate 13-acetate (PMA) plus 500 ng/ml ionomycin (Sigma-Aldrich Chemie, Germany) in the presence of a protein transport inhibitor (Golgi Stop, Pharmingen, San Diego, Calif.) for 5 h in CM/10% (v/v) FCS. Cells were permeabilized, fixed and stained either with APC-labeled IFN- γ -specific or PE-labeled anti-IL-4 mAb or the appropriate isotype controls (FITC-conjugated rat IgG1 κ and PE-conjugated rat IgG1 κ , both clone R3-34) according to standardized protocols (BD Cytotfix/Cytoperm Kit Manual, Pharmingen, San Diego, Calif.).

Immunization of mice

The immature DCs on day 6 were left un-pulsed or were loaded under serum-free conditions in CM with soluble K-Balb-NPT lysate, synthetic NPT-derived peptides or recombinant NPT protein and matured with 100 ng/ml LPS (Sigma-Aldrich Chemie, Germany) and 10 ng/ml IFN- γ (Pharmingen, San Diego, Calif.) for 2 or 24 h to generate active or exhausted DCs, respectively. Cells were harvested (Accutase, PAA, Linz, Austria), washed once, and adjusted to 10×10^6 /ml in 0.9% (w/v) NaCl (Braun, Melsungen AG, Melsungen, Germany) for in vivo applications. In the prophylactic setting, mice were shaved and were injected s.c. 14 and 7 days before tumor challenge with 1×10^6 active un-pulsed or active or exhausted antigen pulsed DCs. Viable K-Balb-NPT tumor cells were injected as a single cell suspension of 0.5×10^6 cells in 100 μ l sterile 0.9% (w/v) NaCl into the opposite flank. Alternatively, to prove a therapeutic benefit in established tumors, tumor-bearing mice were injected s.c. with DCs on day 6, 9 and 12 after tumor cell inoculation. This procedure had been optimized in preliminary experiments, where we found an effect of DC-based immunization only if applied before day 12 after tumor cell challenge (data not shown). Mice treated at later time points (i.e., day 9–12–15 or day 12–15–18) had already developed bulky and rapidly progressing tumors. In addition, initial experiments showed that immunizations could mediate growth delay of established tumors only if applied repeatedly (three times). Control groups received tumor cells without treatment or were injected with NaCl alone. The size of the tumors was measured every 3 to 4 days and recorded as a tumor index (= product of the vertical and horizontal dimension of the tumor in millimeters).

Statistical analysis

For statistical analysis of tumor growth using specific DC treatments, the results of three separate experiments

performed in an identical fashion were combined. The statistical significance was determined by applying two-tailed Student's *t*-test (StatView, version 5.0, Altura Software Incorporations, Anaheim, Calif.) and was defined as $P < 0.05$.

Results

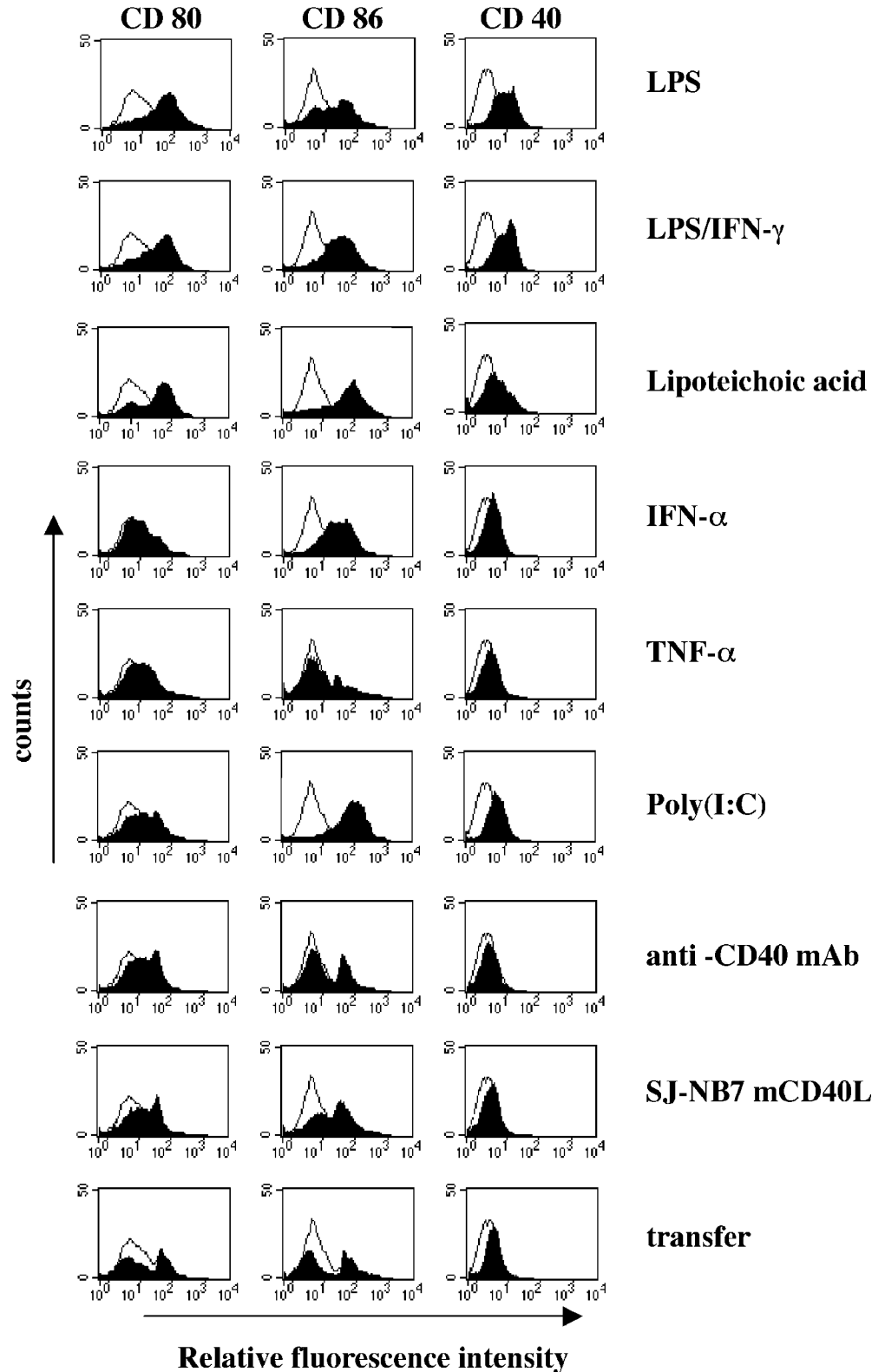
Immunophenotypic characterization of DCs

As a standard procedure ($n > 20$), bone marrow-precursor cells were differentiated into immature DC by a 6-day culture in complete medium containing GM-CSF and IL-4. Generation of immature DCs was confirmed by the expression of CD11b and CD11c ($> 70\%$ of the initial number of progenitor cells). To define optimal conditions for the maturation of DCs, we initially tested several maturation stimuli with respect to their capacity to up-regulate the co-stimulatory molecules CD80, CD86 and CD40. A time period of 24 h of exposure to the maturation agents was chosen to allow optimal maturation while ascertaining full viability of DCs, as a prolonged maturation of DCs results in senescence or apoptosis (unpublished observations). We found that LPS alone or in combination with IFN- γ , or LTA most consistently triggered maturation and yielded phenotypically mature DCs as determined by expression of co-stimulatory molecules CD80, CD86 and CD40 (Fig. 1). Other maturation agents tested, such as TNF- α , IFN- α , Poly (I:C) or cross-linking of CD40 with transgenic SJ-NB7-mCD40L cells induced only lower levels of CD80 and CD40 expression. Mechanical manipulation by a repetitive transfer of immature DCs from well to well had a modest effect on the maturation of DCs as reported previously [10]. Exposure of immature DCs to CD40L induced maturation in only a subpopulation of the cells, suggesting that DCs differentiated from bone marrow precursor cells contain subpopulations unresponsive to CD40/CD40L interaction.

LPS/IFN- γ maturation most effectively triggers IL-12 p70 production by DCs

Since (1) IL-12 secretion has recently been advocated as being stringently correlated with T cell effector function and (2) expression of co-stimulatory molecules is considered to be required for the optimal stimulation of T cells, we tested whether optimally inducing the expression of co-stimulatory molecules would also induce secretion of high levels of IL-12. As shown in Fig. 2a, a combination of LPS with IFN- γ not only was most effective to induce high levels of expression of co-stimulatory molecules, but clearly was superior to all other agents tested for induction of IL-12 p70 release after a 24-h period. Agents that induced lower levels of co-stimulatory molecule expression also induced lower levels of IL-12 secretion.

Fig. 1 Induction of co-stimulatory molecules by maturation of DCs with LPS/IFN- γ . DCs exposed to an array of maturation stimuli for 24 h were analyzed by flow cytometry. The histograms show the expression density of CD80, CD86 and CD40 of immature DCs (*open histograms*) and of mature DCs (*full histograms*). Cells were gated by forward scatter/side scatter profile (*FSC/SSC*). Data are shown as one representative experiment out of three experiments using various maturation stimuli as indicated. Immature DCs were generated for each of the independent experiments from precursor cells from one mouse



Next, we determined the time period of in vitro exposure of immature DCs to the maturation agents LPS/IFN- γ required to obtain both a mature phenotype and high levels of IL-12 secretion. Specifically, we explored whether a short-term exposure of immature DCs

to LPS/IFN- γ would be sufficient to initiate maturation pathways and coordinate IL-12 secretion. In these experiments we compared the phenotype and IL-12 p70 release of (1) DCs exposed to LPS/IFN- γ for 2 h, washed and re-cultured for another 24 h in medium only

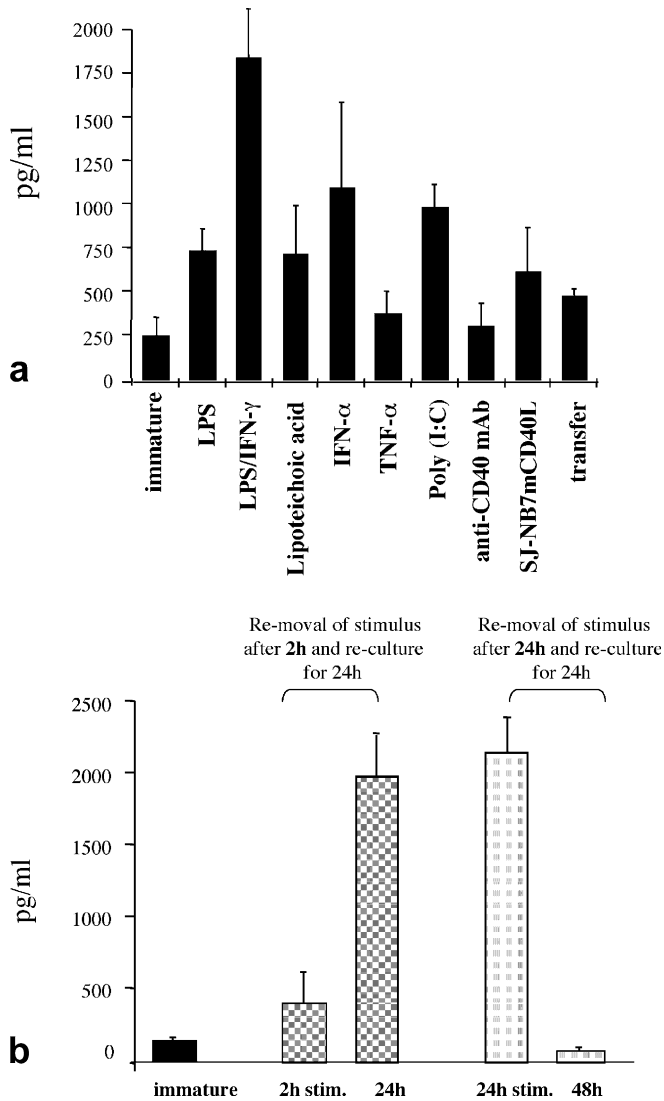


Fig. 2 **a** IL-12 p70 heterodimer production by differently matured DCs. IL-12 production from DCs in response to maturation with the indicated stimuli was determined by ELISA. Values represent cytokine concentrations produced by 0.7×10^6 cells per 1 ml of complete medium (given is the mean \pm SEM of three sequential experiments; DCs were generated from three different animals). **b** Continuing release of IL-12 p70 after a short-time exposure of DCs to LPS/IFN- γ . IL-12 production in the supernatants of DC cultures was tested after a 2-h exposure or a 24-h exposure to LPS/IFN- γ and re-cultivation in complete medium for 24 h (given is the mean \pm SEM of three sequential experiments, DCs were generated from three different animals)

and (2) DCs continuously exposed to LPS/IFN- γ for 24 h. Intriguingly, shortly activated DCs exposed to LPS/IFN- γ for 2 h showed a fully mature phenotype after 24 h re-culture (data not shown). Moreover, as shown in Fig. 2b, in DCs exposed to LPS/IFN- γ for 2 h and re-cultured for a subsequent 24 h in medium only, IL-12p70 secretion reached levels similar to those of DCs exposed to LPS/IFN- γ for the full 24-h period. After that time period, the ability of DCs to continue to release IL-12 was exhausted (Fig. 2b, right). Taken together, these findings suggested that a pulse exposure

(2 h) of immature DCs to LPS/IFN- γ was sufficient to initiate processes of (1) full maturation by phenotype and (2) continuous release of IL-12p70 up to 24 h. Hence, by comparing DCs that retain the capacity to release IL-12p70 and DCs that are exhausted in their capacity for IL-12p70 release, we set a platform to study the role of IL-12p70-secreting DCs with respect to their capacity to generate anti-tumor immune responses.

To exclude any unintentional initiation of DC maturation by the procedure of loading DCs with antigen, we determined both the surface marker expression pattern and the IL-12 secretion levels of DCs after antigen pulsing before being exposed to maturation stimuli. Endotoxin contaminated recombinant NPT protein (endotoxin content 250 ng/mg protein) was incubated prior to loading with polymyxin B sulfate. By this procedure we could completely prevent untimely maturation of DCs. We found first that immature DCs loaded with polymyxin B sulfate-pretreated NPT protein, synthetic NPT-derived peptides or tumor cell lysate still retained a typical immature phenotype (data not shown). Second, immature pulsed and unpulsed DCs were susceptible to maturation displaying a mature phenotype after 24 h (up-regulated CD80, CD86, CD40 and MHC class II molecules) after a 2-h exposure to LPS/IFN- γ followed by a replacement of the stimulus with CM/10% FCS (data not shown). Third, loading of DCs with antigen from all different sources before maturation with LPS/IFN- γ for 2 h had no adverse effect on the final IL-12 p70 secretion. Immature DCs, unpulsed and pulsed, released negligible amounts of IL-12 p70 (121 ± 53 pg/ml and 146 ± 78 pg/ml, respectively). Upon maturation with LPS/IFN- γ for 2 h, unpulsed DCs released $2,198 \pm 498$ pg/ml; when pulsed with polymyxin B sulfate pre-treated NPT, they released up to $1,610 \pm 410$ pg/ml IL-12 p70, with synthetic NPT-derived peptides up to $1,600 \pm 684$ pg/ml IL-12 p70 and with tumor cell derived lysate up to $2,200 \pm 748$ pg/ml IL-12 p70 (data not shown). From these results we concluded that loading of DCs with various sources of antigen before maturation did not interfere with their ability to secrete IL-12 p70.

Induction of protective and therapeutic anti-tumor immunity

To test the hypothesis that the DCs' capacity to release IL-12 has an influence on the outcome of the anti-tumor reactivity, we compared active DCs, i.e., matured with LPS/IFN- γ for 2 h, to exhausted DCs, which were matured for 24 h, in a syngeneic mouse tumor model. Based on our in vitro experiments, DCs applied to animals immediately after a 2-h LPS/IFN- γ exposure presumably would continue to secrete peak levels of bioactive IL-12 p70 in vivo, in contrast to exhausted DCs that would fail to continue to secrete IL-12 after the time when they were applied as a vaccine to animals. This model therefore allowed us to evaluate the influence of

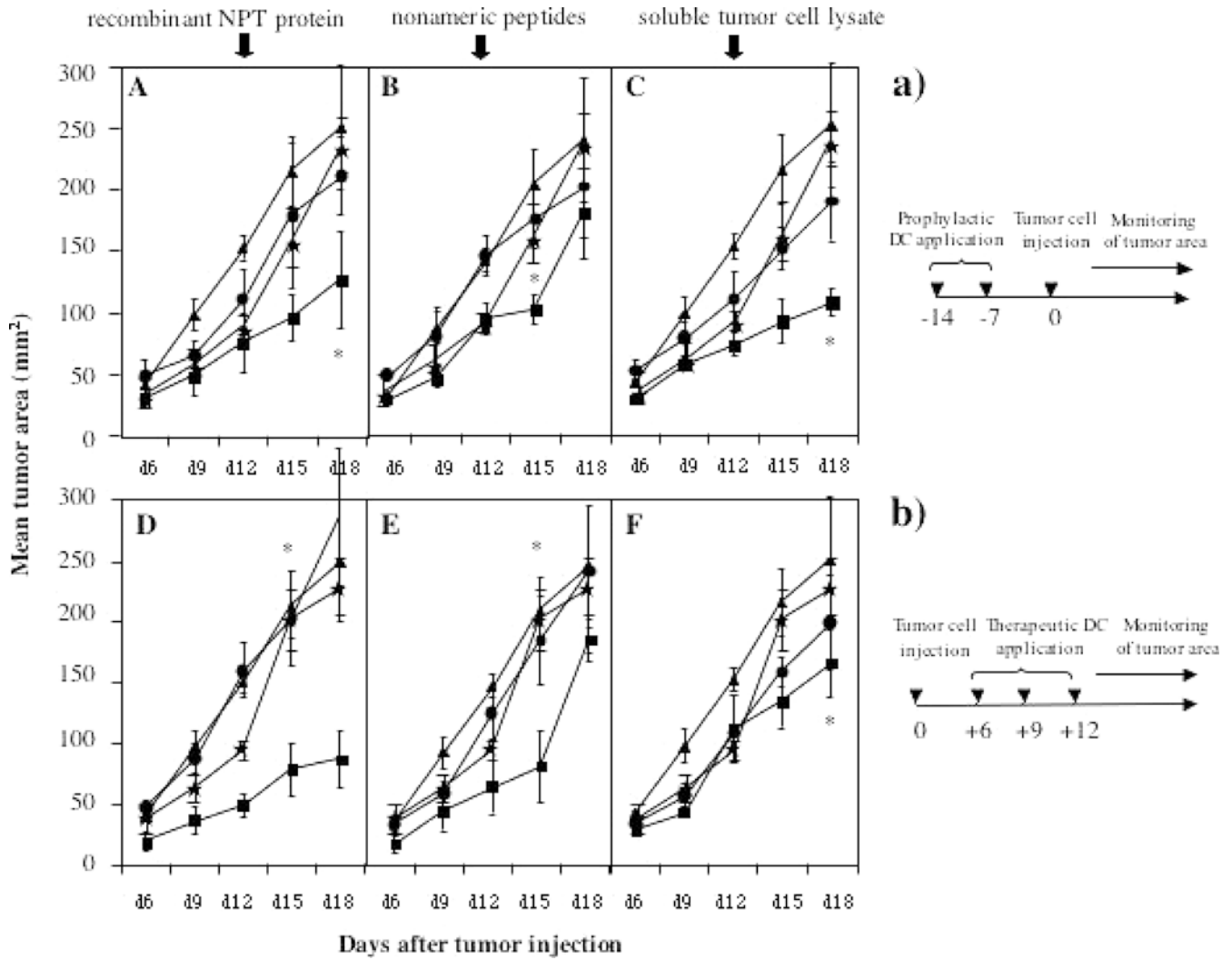


Fig. 3a, b Immunizations with active but not with exhausted DCs trigger anti-tumor immunity. The anti-tumor effect of antigen-loaded DCs was tested in a prophylactic and a therapeutic setting. In the prophylactic setting (**a**) mice ($n=5$) were injected with 1×10^6 active (squares) or exhausted (circles) bone marrow-derived DCs loaded either with recombinant NPT protein (**A**), synthetic NPT-derived peptides (**B**) or K-Balb-NPT lysate (**C**) on days -14 and -7 before being inoculated with 0.5×10^6 K-Balb-NPT tumor cells in the opposite flank on day 0. Tumor growth was monitored up to 20 days after tumor cell inoculation. Tumor size is given as the mean tumor surface area \pm SEM, * $P < 0.05$ (Student's *t*-test), active versus exhausted DCs. (**b**) In the therapeutic setting, mice ($n=5$ per group) received 1×10^6 active or exhausted bone marrow-derived DCs loaded either with recombinant NPT protein (**D**), synthetic NPT-derived peptides (**E**) or K-Balb-NPT lysate (**F**) on day 6, 9 and 12 after tumor cell inoculation. In both settings, control mice received tumor cells only (triangles) or active, but unpulsed DCs (asterisks). The graphs depict one representative experiment out of three independent experiments; each independent experiment used five mice per group

IL-12 production from DCs on the anti-tumor immune responses through our DC-based vaccine.

For prophylactic immunization, naïve syngeneic mice received active un-pulsed or active or exhausted DCs loaded either with recombinant NPT protein, NPT-derived synthetic peptides or soluble K-Balb-NPT-derived

cell lysates twice, at weekly intervals. Control groups were left untreated or received injections with saline alone. One week after the last immunization, mice were challenged by a subcutaneous inoculation of viable K-Balb-NPT cells. After a time period of 18 days, only after a prophylactic immunization with active DCs was a significantly reduced tumor growth observed in mice (Fig. 3a). This protective effect was independent of the tumor antigen source. The reduction in tumor growth of up to 50% was most prominent in mice treated with DCs loaded with NPT-derived protein or soluble tumor cell lysate, but was also significant on day 15 after treatment with NPT-derived peptide-pulsed DCs. In contrast, after treatment with exhausted DCs, tumor growth was similarly aggressive as in untreated control groups, i.e., animals were not protected against tumor growth at all.

In a second set of experiments, active and exhausted DCs were tested for their ability to promote therapeutic anti-tumor immunity. In pilot experiments, we found that therapeutic immunizations in this model can affect tumor growth only if applied before day 12. At later time points, multiple immunizations appeared to have only minimal

effects. After tumor cell inoculation mice were treated with un-pulsed DCs or DCs pulsed with tumor antigens from all three sources at three previously optimized time points as described above (day 6, 9 and 12). Growth of pre-existing tumors was markedly reduced when active, IL-12p70 secreting DCs were applied. Similar to the results from the prophylactic setting, the deceleration of tumor growth was most pronounced when using active DCs pulsed with recombinant NPT protein. Active DCs pulsed with NPT-derived peptides showed an intermediate activity, but still displayed a superior therapeutic potential compared to exhausted DCs ($P > 0.05$ on day 15). When using tumor cell lysate-pulsed DCs, tumor progression was similar to that of control groups (Fig. 3b). Injection of active but un-pulsed DCs could not induce a tumor-specific immune response in both the prophylactic and the therapeutic tumor model. These results demonstrate that the vaccination with active DCs induced significantly greater anti-tumor effects than with exhausted DCs, thus implying that the ability to secrete bioactive IL-12 plays a major requisite role in anti-tumor immunity *in vivo*.

CD4 and CD8 T cells from immunized mice show enhanced IFN- γ secretion when re-stimulated *in vitro* with tumor antigens

Finally, to confirm that active DCs indeed induced T cell activity specific for tumor-associated antigens, we determined the IFN- γ expression from both CD4 and CD8 cells as a marker for T cell activation. Splenocytes of vaccinated mice were either left un-stimulated or were re-challenged *in vitro* with mitomycin C pre-treated K-Balb-NPT tumor cells (Fig. 4). In splenocyte cultures from groups of mice having received active DCs (responding to vaccine and showing efficient control of tumor growth), we found an enhancement of IFN- γ release in CD4 and CD8 populations only in response to tumor cell co-cultivation *in vitro*, but not in cell cultures left un-stimulated (19.4 vs. 8.4% of CD4⁺/IFN- γ ⁺ cells and 11.5 vs. 3.1% CD8⁺/IFN- γ ⁺ cells), which correlated with the enhanced anti-tumor immune response observed *in vivo*. In splenocyte cultures from groups of mice having received exhausted DCs (showing an inefficient anti-tumor activity), we could not detect IFN- γ expressing cell populations in both un-stimulated and previously re-stimulated cells (2.2 vs. 2.4% of CD4⁺/IFN- γ ⁺ cells and 1.9 vs. 2.2% CD8⁺/IFN- γ ⁺ cells). These results provide evidence that active DCs in fact display a potential to (1) present exogenous non-self antigen and to (2) prime T cells effectively against tumor antigen for mobilization of specific immunity.

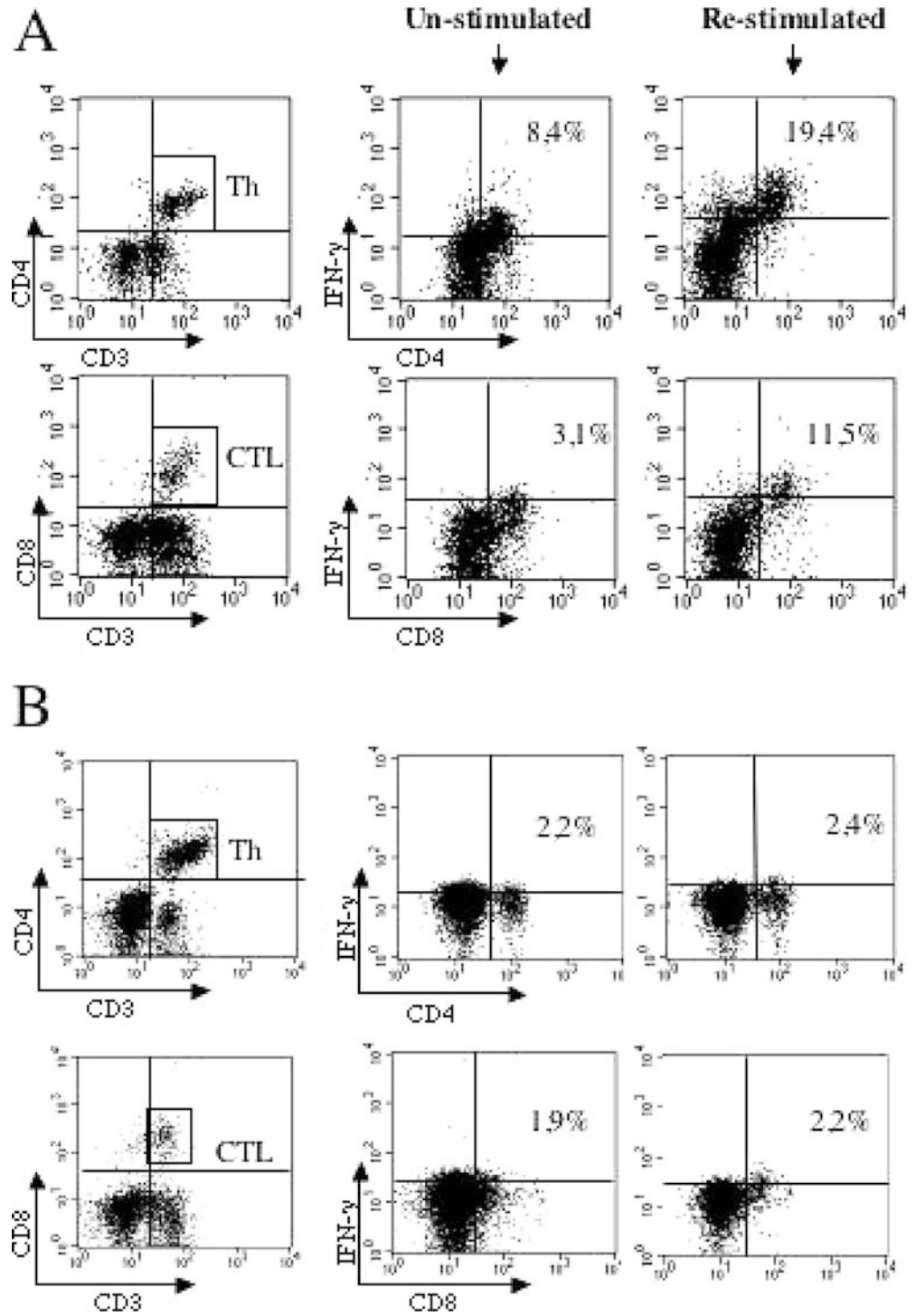
Discussion

The role of different DC types in the generation of cytolytic anti-tumor immune responses is still contro-

versial. DCs, whether they develop from a myeloid or a lymphoid lineage, may differ not only in their phenotype, but also in their location, migratory ability and T cell stimulatory capacity [5, 22, 25, 37] based on different cytokine and transcription factor requirements [1, 12]. Immature DCs have been speculated to mediate peripheral tolerance [6], whereas mature myeloid DCs were suggested to preferentially drive Th1-type responses [21, 37]. As we intended to generate DCs with optimized potential to induce cytolytic effector immune responses, we differentiated DCs from bone marrow cells that clearly include myeloid progenitors. Specifically, we focused on the generation of a stably mature DC phenotype accompanied by the release of IL-12, which is the main Th1-polarizing cytokine. Among the various conditions tested, we found that the initiation of maturation with LPS/IFN- γ for 2 h and subsequent recultivation for 1 day finally resulted in the generation of phenotypically mature DCs that secreted high levels of bioactive IL-12 p70. This implies that, *in vitro*, even after removal of the maturation stimulus, DCs continue to secrete IL-12. Given that these *in vitro* findings correlate to the *in vivo* situation, it is conceivable to speculate that the IL-12 secretion by active DCs contributes to the generation of an effective anti-tumor activity. Continuous cultivation of DCs in the presence of LPS/IFN- γ for 24 h generated DCs that were unable to release IL-12 p70 after removal of the maturation stimulus and were less effective in triggering anti-tumor immunity. This observation clearly warrants careful interpretation of the role of IL-12 in anti-tumor immune responses. Two previous reports suggest that the excellent responsiveness of DCs to a maturation stimulus in the presence of IFN- γ seems to be consistent with a mode of stimulation that mimics the physiological enhancement of IL-12 induction by IFN- γ producing CD4⁺ T cells *in vivo* [15, 31]. In particular, IFN- γ appears to be required for the induction of the p35 subunit of IL-12p70. Furthermore, in the presence of IFN- γ increased antigen presentation because of an up-regulation of the processing and transport machinery and the expression of MHC class I and MHC class II molecules on DCs was demonstrated [36].

The ability of DCs to interact with T cells and to induce a Th1 response is intimately connected with IL-12 by the production of IFN- γ , which in turn favors cytolytic immunity [20, 34]. IL-12 secretion by DCs has been described as following a distinct kinetic: IL-12 is produced from DCs during the first 12–20 h upon initiation of maturation, and during this period it acts as an immune polarizing cytokine favoring polarization to Th1 cells. After 20 h, DCs cease to produce IL-12, and associated with this loss is their switch from a Th1- towards a Th2- polarizing activity [17]. Th2 cells were found to predominantly support antibody production and hence are likely to be of less relevance for anti-tumor immunity. The induction of Th1-based cell-mediated immune responses that include NK cells and CTLs therefore appears to be strictly dependent on the

Fig. 4 a,b Detection of specific Th1 polarized and cytotoxic T cells from immunized mice upon in vitro re-stimulation with tumor cells. Splenocytes from mice, vaccinated as in Fig. 3a, were collected and re-stimulated in vitro with mitomycin C pre-treated K-Balb-NPT tumor cells or were cultured in CM/10% FCS only. On day 5, cells were examined for the expression of IFN- γ by intracellular staining (see Materials and methods). Stimulated (*right panel*) and unstimulated (*middle panel*) CD3⁺/CD4⁺ cells and CD3⁺/CD8⁺ from mice showing delayed tumor growth upon application of DCs (**a**) and from mice that were immunized with exhausted DCs and hence could not control tumor growth (**b**) were analyzed. Numbers indicate the percentage of IFN- γ positive cells in the CD3⁺/CD4⁺ and the CD3⁺/CD8⁺ subpopulations. Given are data from one representative out of three independent experiments; each independent experiment used five mice per groups



cytokine milieu provided during the time period of DC-T cell interaction. As the presence of IL-12 as an immune polarizing signal is critical to accomplish that task, hypothetically, DCs have to be applied early after initiation of maturation to retain the capacity to release IL-12 into the intracellular microenvironment, but not at later time points when their capacity to produce IL-12 is exhausted. In line with this hypothesis, we demonstrate here that the generation of an anti-tumor immune

response by presentation of exogenous tumor antigens requires a distinct functional active DC phenotype that is associated with the secretion of IL-12. The data presented here complement the finding of a most recent study, which described the impact of the DC-IL-12 release and of their subsequent functional changes on the outcome of the tumor-specific immune response [2].

Most significantly, we have shown that DCs continue to secrete IL-12 even after removal of the maturation

stimulus. Such retained capacity may explain the generation of powerful CTLs by shortly activated DCs [2]. Comprehensively viewing these experimental findings underscores the hypothesis that a Th1 polarization occurs within a narrow time window preferentially very soon after initiation of DC maturation, i.e., involves DCs that reach the lymphoid tissues at a time when they are prompted to release IL-12, thus inducing a potent CTL response *in situ*.

In order to trigger CTL-mediated immunity against exogenous antigens, DCs need to receive a licensing signal. Three general mechanisms for licensing have been described. First, DCs can become licensed upon a conditioning signal that is delivered by interaction of the surface molecule CD40 on DCs and its ligand CD40L expressed on T cells [27, 29]. Second, DCs can be licensed to trigger CTLs upon recognition of bacterial cell wall components [23, 30]. Finally, DCs are licensed for triggering cytolytic immunity upon virus-derived signals mediated by type I interferons [9]. Additionally, we suggest that the sustained IL-12 release from active DCs may be of crucial importance to induce cross-presentation and induction of CTL activation.

By utilizing IL-12-secreting active DCs, we could generate anti-tumor immunity using exogenous tumor antigen from various sources, e.g., synthetic tumor antigen-derived peptides, whole tumor cell lysates or recombinant proteins. As actively IL-12 secreting DCs showed an effect in raising anti-tumor immune responses only if pulsed with antigen but not if left un-pulsed, it will be important to determine in future experiments whether the effect of IL-12 secreting DCs is (1) on T cells (e.g., by improving antigen recognition and/or T cell stimulation) or (2) on DCs (e.g., by promoting the efficacy of antigen presentation). Alternatively, the reduced anti-tumor immune effects of active, but un-pulsed DCs might be due to (1) a local antigen uptake below threshold or (2) tumor escape mechanisms. Based on the IFN- γ release observed in re-challenged splenocyte cultures from mice showing decelerated tumor growth after treatment with actively IL-12 secreting DCs, we would like to elaborate in future experiments whether actively IL-12 secreting DCs themselves serve as inducers of specific immunity and not merely as a source of IL-12.

In addition to the type of APC, the nature and concentration of antigen may be important for an effective presentation via MHC class I molecules and a subsequent anti-tumor response. Whereas synthetic peptides can bind directly to MHC molecules on the cell surface and thus do not necessarily need any processing, recombinant NPT protein and whole tumor cell lysate require processing and cross-presentation by the DC. Recombinant proteins were initially preferred for loading in many experimental settings because of the unique epitope diversity they make available for both Th cells and CTLs. However, in some cases a rapid *in vivo* turnover of protein antigens or an inefficient presentation from DCs was suggested to contribute to inconsistent experimental results [35]. One limitation to consider

in this study is the high concentration of NPT protein expression by transgenic tumor cells. The inadequate response seen with tumor lysate pulsed DCs in our experiments may be due to (1) the presence of immunosuppressive factors in the lysate, (2) insufficient concentrations of immunogenic proteins in tumor cell lysate [11] and (3) the possible induction of autoimmune responses to self or normal tissue antigens present in the tumor cell lysate [32]. Synthetic peptides are not generally applicable as tumor antigens due to the variability of the binding affinities to the MHC molecule. It was also reported that peptide-pulsed DCs have a shorter life span, which would impede the induction of specific CTLs [19]. Together, these notions strengthen our view that the kind of immune response depends on (1) the form of antigens and (2) how antigens are displayed by the DC.

In conclusion, we could demonstrate that in a murine tumor model mice were protected against K-Balb-NPT tumor growth only if tumor antigens were presented by short-term activated, IL-12 secreting DCs, as opposed to fully mature DCs, that had exhausted their capacity to produce IL-12. We therefore conclude that with carefully timed *in vitro* maturation using specific stimuli, i.e., LPS and IFN- γ for 2 h, one can induce DCs that display a mature phenotype, produce optimal quantities of IL-12p70 and are therefore enabled to promote specific anti-tumor CTL responses *in vivo*. The value of the current data and the validity of the conclusions highlight the requirement for further testing in patient trials to consider the timing and character of maturation in the design of cancer vaccines and to be mindful of IL-12 exhaustion of DCs.

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