

Reversal of Tumor-induced Dendritic Cell Paralysis by CpG Immunostimulatory Oligonucleotide and Anti-Interleukin 10 Receptor Antibody

Alain P. Vicari,¹ Claudia Chiodoni,² Céline Vaure,¹ Smina Ait-Yahia,¹ Christophe Dercamp,¹ Fabien Matsos,¹ Olivier Reynard,¹ Catherine Taverne,¹ Philippe Merle,³ Mario P. Colombo,² Anne O'Garra,⁴ Giorgio Trinchieri,¹ and Christophe Caux¹

¹Schering-Plough Laboratory for Immunological Research, 69571 Dardilly, France

²Department of Experimental Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milano, Italy

³Institut National de la Santé et de la Recherche Médicale U271, 69424 Lyon, France

⁴DNAX Research Institute, Palo Alto, CA 94304

Abstract

Progressing tumors in man and mouse are often infiltrated by dendritic cells (DCs). Deficient antitumor immunity could be related to a lack of tumor-associated antigen (TAA) presentation by tumor-infiltrating DCs (TIDCs) or to a functional defect of TIDCs. Here we investigated the phenotype and function of TIDCs in transplantable and transgenic mouse tumor models. Although TIDCs could encompass various known DC subsets, most had an immature phenotype. We observed that TIDCs were able to present TAA in the context of major histocompatibility complex class I but that they were refractory to stimulation with the combination of lipopolysaccharide, interferon γ , and anti-CD40 antibody. We could revert TIDC paralysis, however, by *in vitro* or *in vivo* stimulation with the combination of a CpG immunostimulatory sequence and an anti-interleukin 10 receptor (IL-10R) antibody. CpG or anti-IL-10R alone were inactive in TIDCs, whereas CpG triggered activation in normal DCs. In particular, CpG plus anti-IL-10R enhanced the TAA-specific immune response and triggered *de novo* IL-12 production. Subsequently, CpG plus anti-IL-10R treatment showed robust antitumor therapeutic activity exceeding by far that of CpG alone, and elicited antitumor immune memory.

Key words: cancer • immunosuppression • immunotherapy • interleukin 12 • Toll-like receptor

Introduction

Although the main function of dendritic cells (DCs)* is probably to orchestrate a defense against pathogens, DCs are also well equipped to initiate antitumor responses (1). Indeed, *in vitro*-generated DCs are able to sample and present tumor antigens for the priming of cytotoxic T cells (2) and DCs can produce the cytokines IL-12, TNF α , and IFN- α that play diverse roles in antitumor immune

responses (1). Accordingly, several investigators have successfully harnessed this biological potential of DCs by preventing or curing transplantable tumors in mice after the infusion of *ex vivo*-derived DCs pulsed with tumor-associated antigens (TAAs; references 3 and 4). Today, this strategy is being evaluated in clinical trials investigating what is the most efficient subset of DCs, form of antigen, and activation stimulus (5).

Although solid human tumors are frequently infiltrated by DCs (6–8), only a few studies focused on the therapeutic potential of tumor-infiltrating DCs (TIDCs). Yet, in a mouse model, TIDCs were shown to have captured and processed TAA exogenously in the MHC class I pathway (9), suggesting that this first step toward immune reactivity was not impaired. However, the tumor milieu appears to lack the expression of DC activation factors such as microbial

A. O'Garra's present address is The National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

Address correspondence to Alain P. Vicari, Schering-Plough Laboratory for Immunological Research, BP11, 27 chemin des Peupliers, 69571 Dardilly, France. Phone: 33-4-72-17-27-00; Fax: 33-4-78-35-47-50; E-mail: alain.vicari@spcorp.com

*Abbreviations used in this paper: BM-DC, bone marrow-derived DC; DC, dendritic cell; TAA, tumor-associated antigen; TIDC, tumor-infiltrating DC.

stimuli which are known to be crucial in DC physiology (10). Furthermore, tumor cells or tumor-infiltrating cells may produce IL-10, PGE₂, and TGFβ that impair DC functions (11, 12). Thus, lack of activation in conjunction with inhibition of DCs within tumors could explain why the immune response against the tumor is not taking place. Therefore, manipulations aimed at restoring TIDC function may provide novel immunotherapeutic strategies against cancer.

In this report, we observed that TIDCs in several transplantable and transgenic mouse tumor models uniformly had an immature phenotype and were refractory to activation with a combination of microbial and T cell–derived stimuli: LPS, IFN-γ, and anti-CD40 agonist antibody. We then identified conditions that reverted tumor-induced DC paralysis, namely the combination of an immunostimulatory unmethylated CpG oligonucleotide and an anti-IL-10 receptor (IL-10R) antibody. Consequently, the CpG plus anti-IL-10R combination showed robust anti-tumor therapeutic efficacy, exceeding by far that of CpG alone.

Materials and Methods

Mice. Female BALB/c, C57BL/6, and BALB/c × C57BL/6 F1 mice were from Charles River Laboratories. The X/myc transgenic mouse lineage spontaneously develop Hepadnavirus-related hepatocellular carcinoma due to up-regulation of *c-myc* oncogene in the liver (13). Procedures involving animals and their care were conducted in conformity with EEC Council Directive 86/609, OJL 358,1, December 12, 1987.

DC Preparation and Culture. TIDCs or lymph node DCs were purified using CD11c⁺ Microbeads (MiniMacs; Miltenyi Biotec) as described previously (14). DCs were purified from tumors of an average of 0.5 to 0.8 cm in diameter, and the time needed to reach such size varied from 2–4 wk depending on the tumor model. Bone marrow–derived DCs (BM-DCs) were obtained as described (14). When indicated, DCs were activated with combinations of 10 ng/ml LPS (Sigma-Aldrich), 20 ng/ml IFN-γ (R&D Systems), 20 μg/ml purified anti-CD40 antibody (clone FKG45.5, a gift from A.G. Rolink, Basel Institute for Immunology, Basel, Switzerland), 10 μg/ml purified anti-IL-10R antibody (clone 1B1.3a [15]) or control rat IgG1 antibody (clone GL113) and 5 μg/ml phosphothioate-modified CpG 1668 (5'-TCCATGACGTTCCCTGATGCT-3'; MWG-Biotech), or control GpG 1668 (5'-TCCATGAGGTTCCCTGATGCT-3'). In some experiments, a 48 h supernatant made from excised 0.5 to 0.8 cm in diameter C26 tumors was added at 10% vol/vol.

Tumors and In Vivo Procedures. The C26 colon carcinoma (H-2^d) and its variant C26–6CK, engineered to express the murine chemokine CCL21/SLC/6CKine, have been described previously (14). The P815 mastocytoma (H-2^d), LL2 lewis lung carcinoma and B16F0 melanoma (both H-2^b) were purchased from American Type Culture Collection. TSA is a H-2^d mammary carcinoma (16) and MC38 a H-2^b colon carcinoma (17). Tumor cells were injected subcutaneously at day 1 and tumor growth was monitored by palpation and measurement using a caliper three times a week. For treatment, mice were injected at day 7, 14, and 21 with various combinations of intraperitoneal 250 μg anti-IL-10R antibody or control antibody and intra- or peritumoral 5 μg CpG 1668 or control GpG 1668. For cell depletion in vivo, 0.5 mg of anti-CD8 or anti-CD4 (clones 2.43 and GK-1.5; American Type Culture Collection) purified antibodies or 10

μl of rabbit anti-Asialo-GM1 serum (Wako Pure Chemical Industries) were injected twice a week intraperitoneally starting 1 d prior to tumor cell inoculation, then at day 3 and once a week after the first week during the course of the treatment.

Flow Cytometry Analyses. Fc receptors were blocked using Fc-Block™ (BD Biosciences). The antibodies (all from BD Biosciences) used in this study were: CD8α (53–6.7), CD11c (HL3), CD11b (M1/70), CD40 (HM40–3), CD45R/B220 (RA3–6B2), CD86 (GL1), and I-A^d/I-E^d (clone 2G9). Biotinylated antibodies were revealed with PE or PE-Cy5 streptavidin (Dako). For intracellular detection of IL-12p40/p70, cells previously incubated for 2 h in Brefeldin A (Sigma-Aldrich) and stained with CD11c were processed with the Fix & Perm™ kit (Caltag) and stained with PE-labeled anti-IL-12 (clone C15.6; BD Biosciences). Parameters were acquired on a FACScan™ (Becton Dickinson).

Mixed Leukocyte Reaction. T lymphocytes were obtained from C57BL/6 (H-2^b) mice by depletion of lymph node cells with anti-CD19, anti-Ia, anti-CD11b, and anti-TER-119 antibodies with anti-rat immunoglobulin magnetic beads (Dyna). Triplicates of 3 × 10⁵ purified T cells were incubated with various numbers of stimulator cells γ-irradiated (3,000 rad) for 5 d. Proliferative responses were measured by incorporation of [³H]thymidine (Amersham Biotech) for the last 18 h of culture.

Stimulation of C26-specific CTLs In Vitro. TIDCs enriched from BALB/c × C57BL/6 F1 mice bearing C26 tumors and spleen DCs from F1 mice were used to stimulate IFN-γ production by the CTL clone E/88, specific for the murine leukemia virus (MuLV) *env*-derived AH-1 peptide SPSYVYHQF presented by H-2^d, and the CTL line TG905, specific for the MuLV *env*-derived peptide KSPWFTTL presented by H-2^b as described (9, 17, 18).

Cytotoxicity Assay. Spleen cell suspensions were cultured in the presence of 10⁵ γ-irradiated (20,000 rad) C26 cells and 10 ng/ml IL-2 (Sigma-Aldrich). After 5 d of culture, responder cells were incubated in a classical ⁵¹Cr release-cytotoxic assay with P815 target cells previously loaded or not with 1 μM AH-1 peptide and labeled with Na⁵¹CrO₄ (NEN Life Science Products). Specific cytotoxicity was calculated as: % cytotoxicity = 100 × (cpm experimental – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release).

Cytokine Measurements and Numeration of IFN-γ-producing Cells by ELISPOT. Supernatants from the different cultures were collected after 18 h and assayed for IL-12p40, IL-12p70, and TNFα using DuoSet® ELISA antibodies (R&D Systems). ELISPOT 96-well nitrocellulose-bottom plates (Millipore) coated with rat anti-mouse IFN-γ (clone R4–6A2; BD Biosciences) were seeded with 2 × 10⁵ lymph node cells and incubated with 10 ng/ml IL-2 with or without 1 μM AH-1 peptide for 24 h. IFN-γ-producing cells were revealed by sequential incubations with anti-IFN-γ biotinylated antibody (clone XMG1.2; BD Biosciences), peroxidase-streptavidin (Southern Biotechnology Associates, Inc.) and substrate (AEC; Vector Laboratories).

Statistical Analysis. Statistical analysis of results was performed with the Statview software (Abacus Concepts). The χ² test was used to analyze differences between percentages of tumor bearing mice at a given time, the log-rank test was used to analyze curves of tumor development. Student's *t* test was used in other analyses when indicated.

Results

Tumors Are Infiltrated by Immature DCs Refractory to LPS plus IFN-γ plus Anti-CD40 Stimulation. We isolated TIDCs from various transplantable tumors as well as from

hepatocarcinoma developing in *X/myc* transgenic mice (13) and analyzed their phenotype (Fig. 1). TIDCs purified from a subcutaneously implanted C26 colon carcinoma transduced with the 6Ckine chemokine (14) were CD11b⁺, CD8 α ⁻, and B220⁻ in their vast majority (Fig. 1 A). As shown previously, this chemokine-induced model allowed us to recover large numbers of TIDCs, while these were identical to TIDCs isolated from parental C26 tumors. TIDCs isolated from other transplantable tumors had also a similar phenotype, thus resembling the classical myeloid subset of DCs described in the mouse (19). On the other hand, TIDCs isolated from liver hepatocarcinoma were more diverse, including CD11b⁺ and CD11b⁻ DCs as well as cells expressing CD8 α an/or B220, the latter marker being ascribed to mouse type I IFN-producing cells (19, 20). We then compared the expression of MHC class II, CD40, and CD86 molecules of TIDCs to that of lymph node DCs or immature BM-DCs (Figs. 1 B and 2 A). We observed that TIDCs had an immature phenotype, with intermediate levels of surface MHC class II and no detectable CD40 or CD86 molecules, with the exception of the B16 melanoma TIDCs which expressed low levels of CD40 and CD86.

A feature of immature DCs is a response to stimulation with LPS plus IFN- γ plus anti-CD40 antibody by increasing the expression of CD40 and CD86 (Fig. 2 A) as well as by producing IL-12 p70 (Fig. 2 B). In contrast, TIDCs from

C26-6CK tumors maintained a similar phenotype under activation and did not produce detectable IL-12 p70 (Fig. 2). Similarly, DCs from normal liver produced IL-12 p70 in response to LPS plus IFN- γ plus anti-CD40, whereas TIDCs from hepatocarcinoma did not (Fig. 2 B). Last, a supernatant from C26 tumors added at the time of activation abolished the secretion of IL-12 p70 in BM-DCs (Fig. 2 B). These results indicate that tumors induced DCs to be refractory to LPS plus IFN- γ plus anti-CD40 stimulation.

Combination of CpG and Anti-IL-10R Antibody Overcomes TIDCs Paralysis In Vitro. We tested different combinations of substances with the aim of relieving tumor-mediated inhibition and simultaneously mediating DC activation, including combinations of the TLR-9 ligand CpG 1668 (21) and an anti-IL-10R blocking antibody (15). In control BM-DCs, CpG alone was able to induce the secretion of IL-12p70 and TNF α in amounts similar to that obtained with LPS plus IFN- γ plus anti-CD40 (Fig. 3 A). The addition of anti-IL-10R antibody to BM-DC culture increased the production of IL-12p70 by ~30% but did not modify TNF α secretion (Fig. 3 A). Of note, we could not detect endogenous production of IL-10 by BM-DCs (detection limit: 15 pg/ml). An anti-IL-10 antibody also increased IL-12p70 production by BM-DCs (unpublished data), suggesting that indeed IL-10 and not another ligand of IL-10R α was involved and that low levels of autocrine or paracrine IL-10 were probably sufficient to mediate the effect.

Anti-IL-10R alone was not able to restore significant IL-12p70 production by TIDC cultured in the presence of LPS plus IFN- γ plus anti-CD40 (Fig. 3 B), but increased to some extent their ability to produce TNF α as well as their capacity to stimulate allogeneic T cells in MLR (Fig. 3 C). On the other hand, CpG alone did not induce the secretion of IL-12p70 nor TNF α by TIDCs (Fig. 3 B) and minimally improved their function in an MLR (Fig. 3 C). In marked contrast, the combination of CpG and anti-IL-10R antibody induced the secretion by TIDCs of large quantities of IL-12p70 as well as TNF α (Fig. 3 B) and was also the best activation condition for MLR (Fig. 3 C), although we did not observe a significant increase in CD40 or CD86 expression after activation (unpublished data). TIDCs produced 0.95 ± 0.39 ng/ml IL-10 when stimulated with LPS plus IFN- γ plus anti-CD40 and 2.36 ± 0.62 ng/ml IL-10 when stimulated with CpG. Thus, we identified the combination of CpG plus anti-IL-10R antibody as a novel and unique way to activate TIDCs from C26-6CK tumors.

CpG 1668 plus Anti-IL-10R Combination Activates TIDCs In Vivo. We injected C26-6CK tumor-bearing mice with CpG intratumorally and/or with anti-IL-10R antibody intraperitoneally in an attempt to activate TIDCs in vivo (Fig. 4). We observed that CpG plus anti-IL-10R induced the secretion of intracellular IL-12 p40/70 in a large proportion of TIDCs as soon as 2 h after treatment, whereas CpG had a minimal effect and anti-IL-10R alone had no effect.

Stimulation of TIDCs with CpG plus Anti-IL-10R Improves TAA-specific Responses In Vivo. To test whether TIDCs from C26-6CK tumors had been able to capture and process TAAs in the MHC class I pathway, we ex-

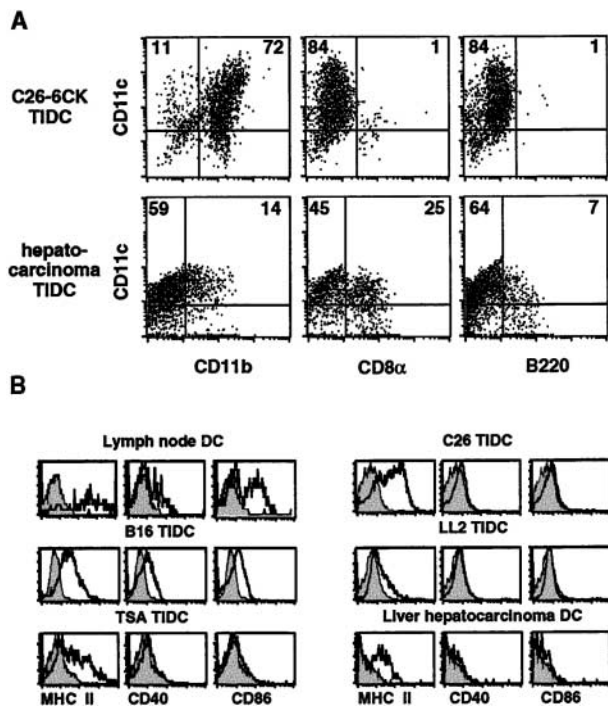


Figure 1. TIDCs may express diverse phenotypes but are immature in their majority. (A) DCs were enriched from the indicated solid tumors and analyzed for the expression of CD11c, CD11b, CD8 α , and B220 by flow cytometry (log scale). (B) DCs were enriched from peripheral lymph node or from the indicated solid tumors and the expression of MHC class II, CD40, and CD86 molecules was analyzed among gated CD11c⁺ cells (solid line) compared with isotype control (gray histograms) by flow cytometry (log scale). Data are representative of two to five experiments.

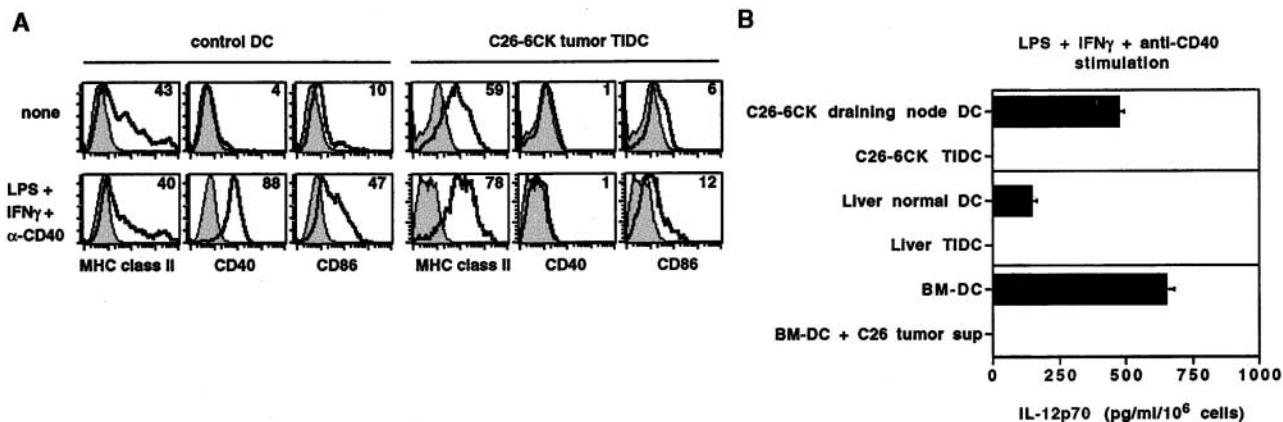
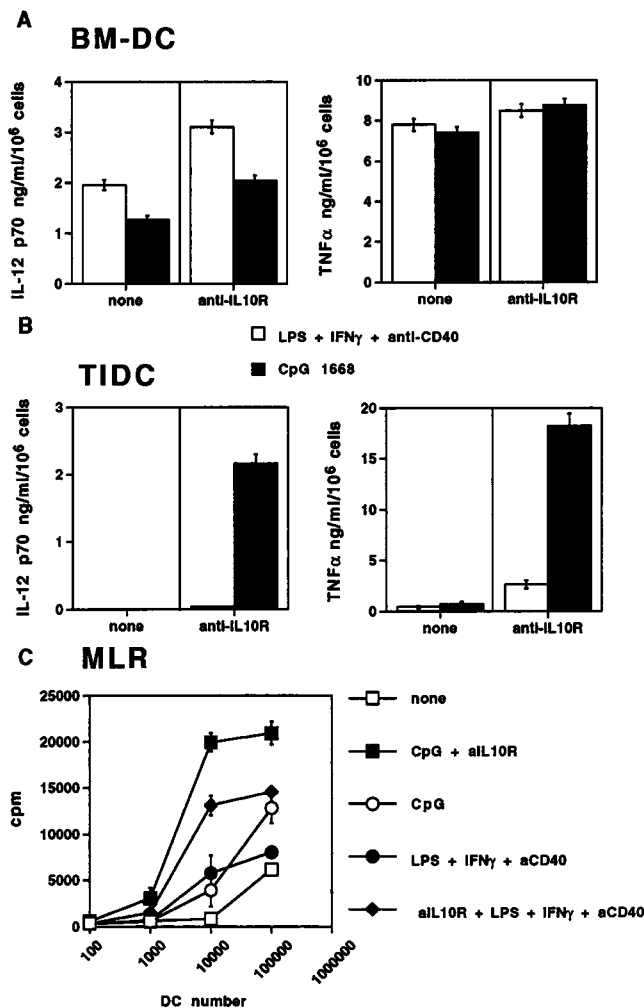


Figure 2. TIDCs are not activated by LPS plus IFN- γ plus anti-CD40 stimulation. (A) Control BM-DCs or enriched C26-6CK TIDCs were cultured overnight with medium alone or with the combination of LPS, IFN- γ plus anti-CD40 antagonist antibody. Flow cytometry histograms (log scale) show the expression of MHC class II, CD40, and CD86 among gated CD11c⁺ cells (solid lines) in unstimulated (top panels) or stimulated (bottom panels) DC populations. The percentage of positive cells was determined by comparison with isotype control (gray histogram) and is indicated in the top right corner of each histogram. Results are representative of more than five experiments. (B) The ability to respond to LPS plus IFN- γ and anti-CD40 activation was analyzed by measuring IL-12p70 levels in 18 h culture supernatants in various DC populations: DCs isolated from C26-6CK tumors or axillary draining lymph nodes from the same tumor-bearing animals; DCs enriched from hepatocarcinoma developing in *x/myc* transgenic mice or from normal liver; BM-DCs cultured in the presence or absence of supernatant from C26 tumors. ELISA results are expressed as the mean concentration \pm SEM of triplicate cultures. Similar results were obtained in two to five experiments, depending upon the conditions tested.



amined their capacity to present TAA-derived peptides to CTL, as described previously for a C26 tumor engineered to express GM-CSF and CD40L (9; Fig. 5 A). The C26 (H-2^d) colon carcinoma expresses an immunodominant TAA which contains the L^d-restricted peptide AH-1 (18), recognized by the E/88 CTL clone. The MC38 (H-2^b) colon carcinoma expresses the same TAA and contains a K^b-restricted peptide recognized by the TG905 CTL line (17). Stimulation of E/88 or TG905 cells by C26 and MC38 cells, respectively, induced IFN- γ production in an MHC-restricted fashion (Fig. 5A). TIDCs purified from BALB/c \times C57BL/6 (H-2^{dxb}) F1 mice bearing C26-6CK tumors were able to stimulate both CTL lines in a cell dose-dependent fashion, whereas spleen DCs purified from naive H-2^{dxb} F1 mice stimulated IFN- γ production only when pulsed with the relevant peptide (Fig. 5 A). In particular, the stimulation of the H-2^b TG905 CTL clearly indicates that TIDCs have been able to capture and present exogenous TAA in the MHC class I pathway.

Figure 3. Combination of CpG 1668 and anti-IL-10R antibody overcome TIDC paralysis in vitro. (A and B) BM-DCs and TIDCs enriched from C26-6CK tumors were activated overnight with either LPS, IFN- γ , and anti-CD40 (white bars) or CpG 1668 (black bars) in the presence (anti-IL-10R) or absence (none) of anti-IL-10R antibody. Culture supernatants were assayed for IL-12p70 and TNF α content. Results are expressed as the mean concentration \pm SEM of triplicate cultures and are representative of more than three experiments. (C) Mixed leukocyte reaction. Irradiated populations of enriched TIDCs, previously activated overnight with none (\square), CpG 1668 (\circ), CpG plus anti-IL-10R (\blacksquare), LPS plus IFN- γ plus anti-CD40 (\bullet), or anti-IL-10R plus LPS plus IFN- γ plus anti-CD40 (\blacklozenge), were cultured with allogeneic purified T cells. Proliferation is expressed as the mean cpm incorporation \pm SEM for triplicates.

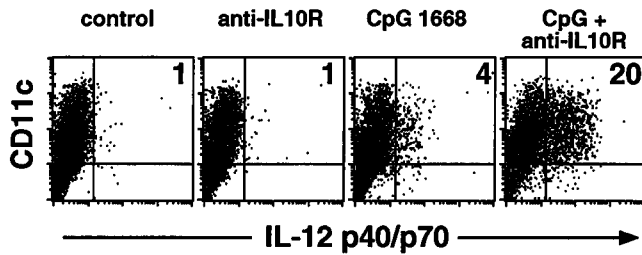


Figure 4. CpG 1668 plus anti-IL-10R activates tumor-infiltrating DCs in vivo. Mice bearing C26-6CK tumors were injected intratumorally with 5 μ g CpG 1668 or control CpG sequence and/or intraperitoneally with 250 μ g anti-IL-10R or isotype control antibody. 2 h after injection, mice were killed, and TIDCs enriched and stained for intracellular IL-12 p40/p70 and surface CD11c. The percentage of IL-12-positive cells among CD11c-enriched TIDCs was determined by comparison with isotype control and is indicated in the top right corner.

We then analyzed the capacity of TIDCs from C26-6CK tumors to induce TAA-specific responses in vivo (Fig. 5, B and C). After enrichment and overnight activation, we injected TIDC to naive mice and 5 d later we measured the AH1-specific response. We found that TIDC from C26-6CK tumors were able to induce AH-1-specific cytotoxicity (Fig. 5 B) as reported previously for C26-GM-CD40L tumors (9). Activation of TIDCs with CpG plus anti-IL-10R did not significantly increase the AH-1-specific cytotoxicity (Fig. 5 B). We observed, however, that only TIDCs activated with CpG plus anti-IL-10R were able to induce significant numbers of AH-1-specific IFN- γ -producing cells (Fig. 5 C). The number of non-AH-1 specific IFN- γ -producing cells was also increased over controls, and we ascribed this data to the fact that the T cell activation triggered by the injection of CpG plus anti-IL-10R-activated TIDCs 5 d before might still be present and include AH-1-specific as well as nonspecific effector cells. Collectively, these results strongly suggest that activation with CpG plus anti-IL-10R antibody increases TAA-specific immune responses.

The Refractory State of TIDC to LPS plus IFN- γ plus Anti-CD40 Activation and Their Sensitivity to CpG plus Anti-IL-10R Are Not Restricted to C26-6CK Tumors. We analyzed CD11c and IL-12 p40/p70 intracellular expression after overnight activation with either LPS plus IFN- γ plus anti-CD40 or CpG plus anti-IL-10R in TIDCs enriched from C26, B16F0, LL2, and TSA transplantable tumors as well as from X/myc hepatocarcinoma (Fig. 6 A). As for C26-6CK TIDCs, DCs isolated from these tumors did not respond to LPS plus IFN- γ plus anti-CD40 but showed a robust response to CpG plus anti-IL-10R (Fig. 6 A). Identical results were obtained by measuring IL-12p70 levels in culture supernatants, albeit we detected minimal IL-12p70 secretion by B16F0 melanoma TIDCs activated with LPS plus IFN- γ plus anti-CD40 (Fig. 6 B). Thus, the refractory state of TIDCs to LPS plus IFN- γ plus anti-CD40 activation and their sensitivity to CpG plus anti-IL-10R can be observed in tumors from different histological origin as well as in a transgenic tumor model.

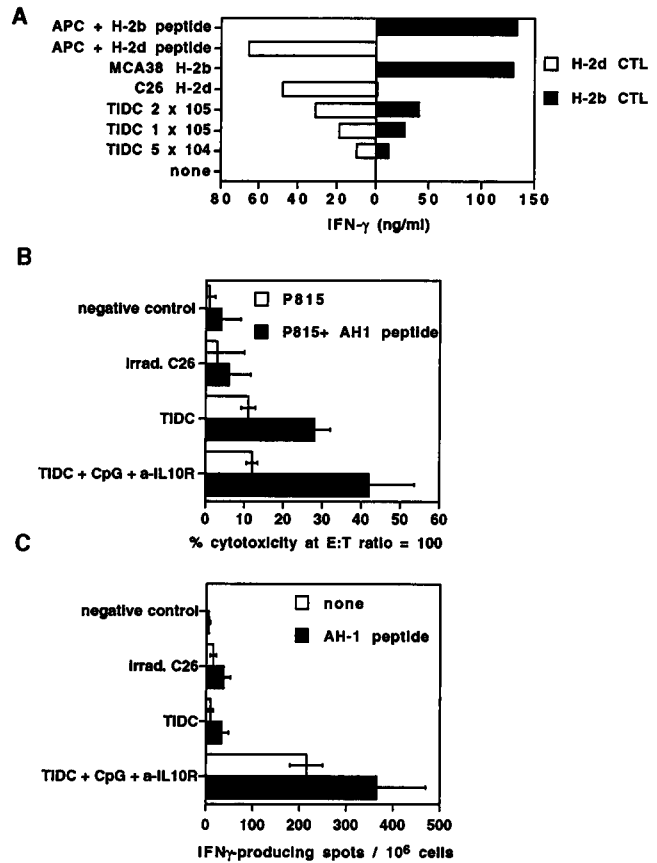


Figure 5. Induction of tumor antigen-specific immune responses by TIDCs and modulation by CpG plus anti-IL-10R. (A) TIDC cross-present tumor antigen-derived peptides to T cells. The ability of TIDCs from C26-6CK tumors grown in H-2^d \times H-2^b F1 mice to present tumor-derived antigenic peptides was assessed by measuring the secretion of IFN- γ by the CTL clone E/88 (H-2^d CTL, white bars) and CTL cell line TG905 (H-2^b CTL, black bars). Positive controls consisted of DCs enriched from H-2^d \times H-2^b spleens (APC) and pulsed with the relevant peptide(s) as well as MCA38 H-2^b and C26 H-2^d cell lines which both express the antigen. (B and C) TIDCs stimulated with CpG 1668 plus anti-IL-10R induce tumor-associated antigen MHC class I-restricted responses in vivo. TIDCs enriched from C26-6CK tumors were cultured overnight with medium alone (TIDC) or CpG 1668 plus anti-IL-10R antibody (TIDC + CpG + a-IL10R), then injected intracutaneously into naive mice. Controls consisted of uninjected mice (negative control) or mice injected with irradiated C26 cells (irrad. C26). Mice were killed 5 d after injection. Experiments were performed with organs pooled from three naive mice and similar results obtained in two separate experiments. (B) Spleens from injected animals were cultured with irradiated C26 cells and IL-2 and cytotoxicity measured against P815 H-2^d target cells, alone (white bars) or loaded with the AH-1 antigenic peptide of C26 (black bars). Results are expressed as the mean cytotoxicity \pm SEM per triplicate wells at an effector/target ratio of 1:100. (C) Total cell suspensions from pooled draining popliteal lymph nodes were analyzed for IFN- γ -producing cells after overnight culture without (white bar) or with (black bar) AH-1 peptide. Results are expressed as the mean number of spots \pm SD for six different wells.

Combination of CpG and Anti-IL-10R Antibody Has Therapeutic Antitumor Effect In Vivo. We treated mice implanted with subcutaneous C26 or B16F0 tumors with various combinations of CpG 1668, control GL113 antibody, or anti-IL-10R antibody (Fig. 7, A and B). We

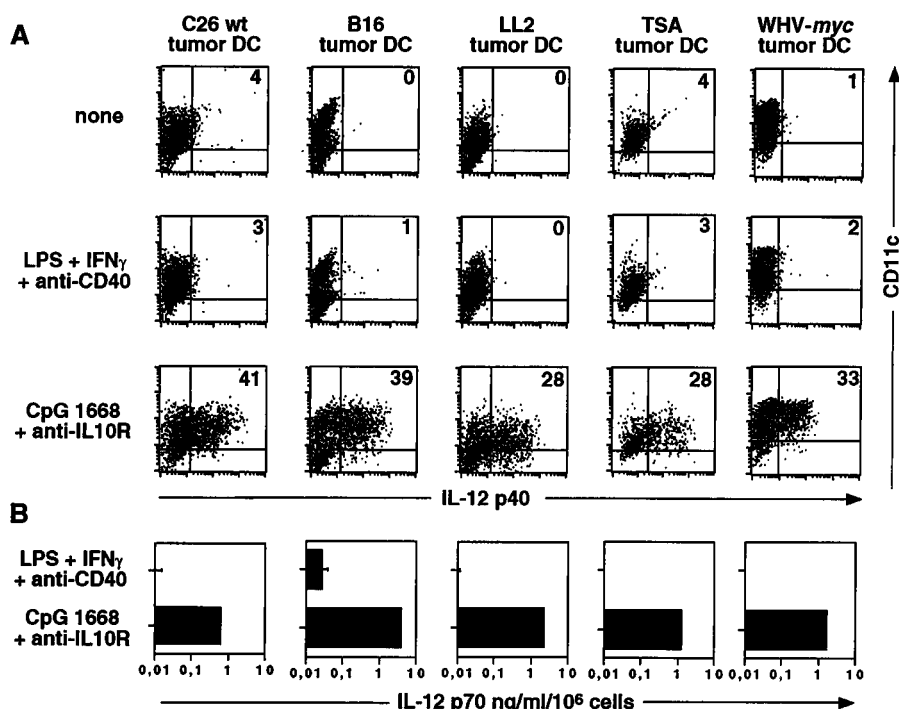


Figure 6. DCs from various transplantable or transgene-induced tumors are refractory to activation with LPS, IFN- γ plus anti-CD40 but not with CpG 1668 plus anti-IL-10R. The ability of T1DC populations enriched from the indicated tumors to produce IL-12 in response to LPS plus IFN- γ and anti-CD40 or CpG 1668 plus anti-IL-10R was analyzed: (A) by intracellular staining for IL-12p40/p70 together with surface CD11c staining (log scale), percentages of CD11c⁺ cells expressing intracellular IL-12 are indicated in the top right quadrant; (B) by measuring IL-12 p70 levels in T1DC culture supernatant by ELISA. Results (log scale) are expressed as the mean concentration \pm SEM from triplicate cultures.

observed that anti-IL-10R or CpG alone had no effect on tumor incidence or survival in the C26 model, nor on tumor incidence in the B16F0 model (Fig. 7, A and B). Treatment with CpG alone had a weak effect on survival in the B16F0 model (Fig. 7 B). In marked contrast, combination of CpG plus anti-IL-10R had a significant effect on tumor incidence ($P = 0.01$ for C26 and $P = 0.05$ for B16F0 compared with control by χ^2 test) and on survival ($P = 0.002$ for C26 and $P = 0.006$ for B16F0 by logrank analysis) in both models (Fig. 7, A and B). We also found similar results in a model where C26-6CK tumors were implanted (unpublished data). Of note, most of the mice treated with CpG plus anti-IL-10R did show palpable tumors, but many developed local necrosis and eventually rejected the tumor.

Therapeutic Effect of CpG plus Anti-IL-10R Depends on Innate and Adaptive Immune Effector Cells. In several additional groups of C26 tumor-bearing mice inoculated with C26 tumor cells and treated with anti-IL-10R plus CpG, we injected antibodies in order to deplete CD4⁺ or CD8⁺ cells or NK cell activity (Fig. 8 A). Although CpG plus anti-IL-10R antibody induced some level of tumor rejection in depleted groups, the combination was less efficient than in nondepleted groups. All mice which rejected the tumor remain tumor-free for the rest of the experiments while those which developed tumors were killed between 4 to 6 wk for antibody-depleted groups and 3 to 5 wk for SCID mice. These data suggest that several components of the adaptive and innate immune response, including CD4⁺, CD8⁺ T cells, and NK cells, might contribute to tumor eradication. We also treated C26 tumor-bearing SCID mice, which lack T

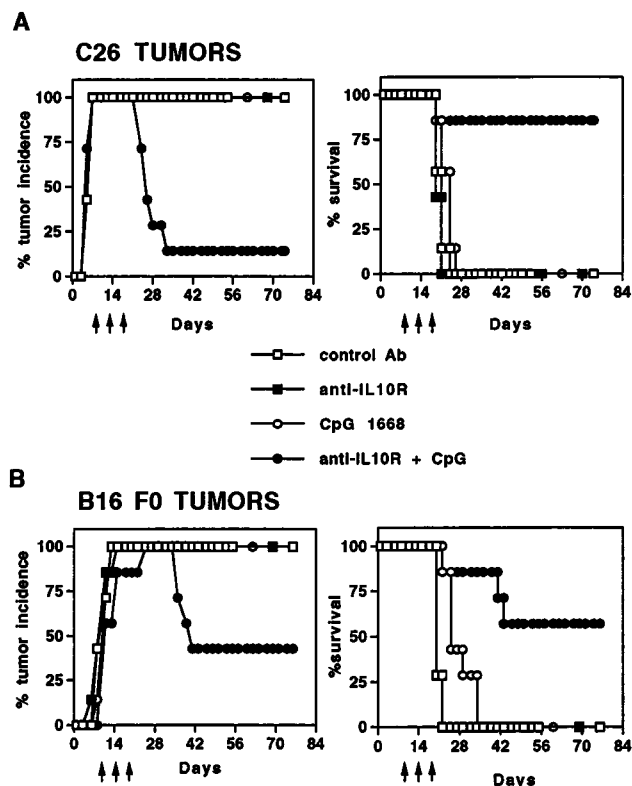


Figure 7. Treatment with CpG 1668 plus anti-IL-10R induces tumor rejection. Groups of seven mice were injected subcutaneously at day 0 with 5×10^4 C26 (A) or B16F0 (B) tumor cells. Mice were treated at day 7, 14, and 21 (arrows) with control antibody, 5 μ g CpG 1668 injected intratumorally, 250 μ g anti-IL-10R antibody intraperitoneally, or CpG plus anti-IL-10R. Tumor incidence and survival were monitored in all groups for the indicated times.

and B cells (Fig. 8 B). There was no significant impact of the treatment on tumor incidence and survival, strongly suggesting a role for T cell and perhaps B cells in the therapeutic effect of CpG plus anti-IL-10R in immunocompetent mice.

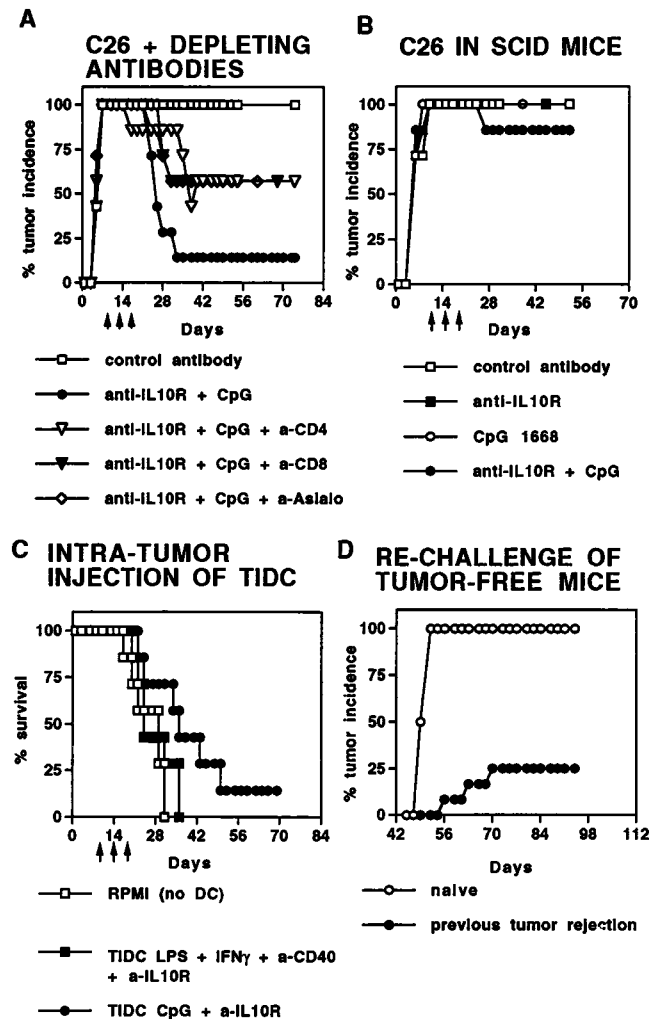


Figure 8. Treatment with CpG 1668 plus anti-IL-10R induces T cell and NK cell-mediated tumor rejection as well as antitumor immune memory. Groups of seven mice were injected subcutaneously at day 0 with 5×10^4 C26 tumor cells. (A) Mice were treated at day 7, 14, and 21 (arrows) with control or 5 μ g CpG 1668 injected intratumorally plus 250 μ g anti-IL-10R antibody intraperitoneally. Indicated groups of mice receiving anti-IL-10R plus CpG were further treated with anti-CD4, anti-CD8, or Asialo-GM1 depleting antibodies as described in Materials and Methods. (B) Immunodeficient SCID mice were treated at day 7, 14, and 21 (arrows) with control, 5 μ g CpG 1668 injected intratumorally, 250 μ g anti-IL-10R antibody intraperitoneally or CpG plus anti-IL-10R. (C) Mice were injected intratumorally at day 7, 14, and 21 (arrows) with 5×10^4 enriched tumor-infiltrating DCs activated for 2 h in vitro with none, LPS plus anti-CD40 plus anti-IL-10R, or CpG plus anti-IL-10R, as well as intraperitoneally with 250 μ g anti-IL-10R antibody. (D) Mice treated with CpG plus anti-IL-10R that had rejected a C26 tumor inoculated at day 0 ($n = 12$) were rechallenged at day 45 with 5×10^4 C26 cells in the contralateral flank and compared naive mice challenged with the same inoculum. Tumor incidence and survival were monitored in all groups for the indicated times.

Therapeutic Effect of CpG plus Anti-IL-10R Can Be the Result of TIDC Activation and Results in Immune Memory. Local treatment with CpG and systemic treatment with anti-IL-10R antibody could have an effect outside of the tumor or act on tumor-infiltrating cells different from DCs. To analyze the direct contribution of activated TIDCs to the antitumor effect, we purified TIDC from C26-6CK tumors, activated them in vitro for 2 h, and then reinjected them intratumorally into C26 tumor-bearing mice (Fig. 8 C). The mice were additionally treated with intraperitoneal injections of anti-IL-10R antibody. We observed a significant increase in survival ($P = 0.03$ by logrank analysis) in mice injected by TIDCs activated with CpG plus anti-IL-10R compared with TIDCs activated with LPS plus IFN- γ plus anti-CD40 plus anti-IL-10R or resting TIDCs (Fig. 8 C). This result suggests that CpG plus anti-IL-10R can promote DC-mediated antitumor effect.

Last, we analyzed the anti-tumor immune memory response in mice cured of C26 tumors with CpG plus anti-IL-10R treatment by rechallenging the animals with C26 cells, 45 d after the first challenge (Fig. 8 D). We observed that only 25% of the mice developed tumors, and with a marked delay when compared with naive mice, suggesting the establishment of an anti-tumor immune memory response after CpG plus anti-IL-10R therapy.

Discussion

Impaired DC functions could explain poor immune reactivity against tumors (for a review, see reference 12). The presence of refractory TAA-loaded immature dendritic cells within tumors could lead not only to the absence of an efficient antitumor response but also to the induction of a tolerogenic response. Indeed, it was recently reported that immature DC injection would induce antigen-specific T cell unresponsiveness in man (22). It was also shown that IL-10 expressed within tumors could promote the generation of tolerogenic regulatory T cells (23). Thus, finding pharmacological means to properly induce TIDC activation could define a novel strategy to cure cancer.

We first observed that DCs from transplantable tumors had an uniform phenotype of classical CD8 α^+ CD11b $^+$ myeloid DCs (19). TIDCs from C26-6CK tumors did not either express CD205 or CD4 (unpublished data), although lack of CD4 expression could have resulted from in situ down-regulation, a phenomenon we observed in tumor-infiltrating CD4 T cells (unpublished data). In contrast, TIDCs from *X/myc* hepatocarcinoma were representative of distinct subsets of mouse DCs (19). It is therefore possible that transplantable tumors are biased in their recruitment of TIDCs. Nonetheless, as we observed that TIDCs from *X/myc* hepatocarcinoma or normal bone marrow or spleen DCs cultured in the presence of tumor supernatant were functionally inhibited (Fig. 2, and unpublished data), we do not think that the TIDC refractory state we describe herein is restricted to a particular subset of DCs. The inability of TIDCs to respond to LPS could not be ascribed to a lack of TLR4 mRNA ex-

pression (unpublished data). To some extent, TIDCs were not completely refractory either to CD40-mediated or to IFN- γ -R signaling, as anti-CD40 antibody as well as IFN- γ further increased IL-12p70 secretion in response to CpG plus anti-IL-10R (unpublished data).

We found that the refractory state of TIDCs was likely induced by soluble tumor-derived factor(s), as supernatant fluid from C26 tumors could similarly paralyze BM-DCs. The ability to induce TIDC activation with the combination of anti-IL-10R plus CpG, as well as with an anti-IL-10 antibody plus CpG (unpublished data), strongly suggests that IL-10 was one of the factors responsible for the induction of the refractory state in vivo, as already described for tumor-associated macrophages (24). Indeed, the C26 tumor supernatant used in the present study contained 240 pg/ml IL-10, as measured by ELISA. Of note, TIDCs from our tumor models did not respond to CpG alone either, in contrast with regular DCs. It was only when anti-IL-10R was added that CpG induced a strong activation of TIDCs. Therefore, as already shown by our previous report (15), it seems that anti-IL-10R antibody can strongly potentiate a microbial stimulus, in particular mediated through a Toll-like receptor. In that particular case, it overcame the unresponsiveness resulting from soluble antigen administration (15). Moreover, it is possible that some pathways of activation downstream of TLRs are completely shut down in TIDCs while others are still operative, as CpG but not LPS was efficient in combination with anti-IL-10R. Other observations have already supported divergent activation pathways via TLR4 and TLR9 (25, 26). The study of TLR-related activation pathways still operative in TIDCs could define novel targets for cancer therapy. Furthermore, it will be of important to test whether our observations also apply to human tumor-infiltrating DCs. Interestingly, a population of plasmacytoid DC precursors has recently been described in human ovarian cancer (27) and these cells, in normal individuals, express TLR9 and respond to selected CpGs (28). Thus, it would be extremely relevant to examine TIDC response to CpG sequences in human TIDCs.

We last found that the CpG plus anti-IL-10R combination had a potent therapeutic anti-tumor effect and induced immune memory. CpG immunostimulatory sequences had been shown to be a potent adjuvant of antitumor immune responses, primarily when mixed with a source of TAA (29–31). Repeated high-dose peritumoral or intraperitoneal injection of CpG could also lead to tumor rejection in the B16 model (32, 33). We found, however, that the combination of CpG plus anti-IL-10R was far more effective than CpG alone. Direct injection of ex vivo activated DCs also had an antitumor effect, albeit to a lesser extent, suggesting that activated TIDCs is a major component of CpG plus anti-IL-10R therapy. The better effect of CpG plus anti-IL-10R therapy when compared with intratumoral TIDC reinfusion could be related to the additional activation of extratumoral DCs. In addition, DCs injected intratumorally may not find the proper way to migrate and/or exert their beneficial effect in situ. When we in-

jected labeled DCs into the tumor and try to recover them the day after, we found very few cells either in the tumor or in the draining lymph node or spleen, suggesting an important cell death during the procedure (unpublished data). In conclusion, our data suggest that using a microbial stimulus by itself may not be effective in some cancers, if DCs that are normally responding to it become refractory because of tumor-derived factors. We postulate that using antagonists of tumor inhibitory factors such as anti-IL-10R may significantly lower the threshold required for TIDC activation to a point amenable for successful immunotherapy using biological modifiers such as CpG sequences.

C. Chiodoni and M.P. Colombo are supported by a grant from the Italian Association for Cancer Research.

Submitted: 6 May 2002

Revised: 11 July 2002

Accepted: 17 July 2002

References

1. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature*. 392:245–252.
2. Ronchetti, A., P. Rovere, G. Iezzi, G. Galati, S. Heltai, M.P. Protti, M.P. Garancini, A.A. Manfredi, C. Rugarli, and M. Bellone. 1999. Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. *J. Immunol.* 163:130–136.
3. Mayordomo, J.I., T. Zorina, W.J. Storkus, L. Zitvogel, C. Celluzzi, L.D. Falo, C.J. Melief, S.T. Ildstad, W.M. Kast, A.B. Deleo, and M.T. Lotze. 1995. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat. Med.* 1:1297–1302.
4. Paglia, P., C. Chiodoni, M. Rodolfo, and M.P. Colombo. 1996. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J. Exp. Med.* 183:317–322.
5. Dallal, R.M., and M.T. Lotze. 2000. The dendritic cell and human cancer vaccines. *Curr. Opin. Immunol.* 12:583–588.
6. Enk, A.H., H. Jonuleit, J. Saloga, and J. Knop. 1997. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int. J. Cancer.* 73:309–316.
7. Bell, D., P. Chomarat, D. Broyles, G. Netto, G.M. Harb, S. Lebecque, J. Valladeau, J. Davoust, K.A. Palucka, and J. Banchereau. 1999. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J. Exp. Med.* 190:1417–1426.
8. Scarpino, S., A. Stoppacciaro, F. Ballerini, M. Marchesi, M. Prat, M.C. Stella, S. Sozzani, P. Allavena, A. Mantovani, and L.P. Ruco. 2000. Papillary carcinoma of the thyroid: hepatocyte growth factor (HGF) stimulates tumor cells to release chemokines active in recruiting dendritic cells. *Am. J. Pathol.* 156:831–837.
9. Chiodoni, C., P. Paglia, A. Stoppacciaro, M. Rodolfo, M. Parenza, and M.P. Colombo. 1999. Dendritic cells infiltrating tumors cotransduced with granulocyte/macrophage colony-stimulating factor (GM-CSF) and CD40 ligand genes take up and present endogenous tumor-associated antigens, and prime naive mice for a cytotoxic T lymphocyte response.

- J. Exp. Med.* 190:125–133.
10. Reis e Sousa, C. 2001. Dendritic cells as sensors of infection. *Immunity*. 14:495–498.
 11. Chouaib S., C. Asselin-Paturel, F. Mami-Chouaib, A. Caignard, and J.Y. Blay. 1997. The host-tumor immune conflict: from immunosuppression to resistance and destruction. *Immunol. Today*. 18:493–497.
 12. Vicari, A.P., C. Caux, and G. Trinchieri. 2002. Tumor escape from immune surveillance through dendritic cell inactivation. *Semin. Cancer Biol.* 12:33–42.
 13. Terradillos, O., O. Billet, C.A. Renard, R. Levy, T. Molina, P. Briand, and M.A. Buendia. 1997. The hepatitis B virus X gene potentiates *c-myc*-induced liver oncogenesis in transgenic mice. *Oncogene*. 14:395–404.
 14. Vicari, A.P., S. Ait-Yahia, K. Chemin, A. Mueller, A. Zlotnik, and C. Caux. 2000. Antitumor effects of the mouse chemokine 6Ckine/SLC through angiostatic and immunological mechanisms. *J. Immunol.* 165:1992–2000.
 15. Castro, A.G., M. Neighbors, S.D. Hurst, F. Zonin, R.A. Silva, E. Murphy, Y.J. Liu, and A. O'Garra. 2000. Anti-interleukin 10 receptor monoclonal antibody is an adjuvant for T helper cell type 1 responses to soluble antigen only in the presence of lipopolysaccharide. *J. Exp. Med.* 192:1529–1534.
 16. Allione, A., M. Consalvo, P. Nanni, P.L. Lollini, F. Cavallo, M. Giovarelli, M. Forni, A. Gulino, M.P. Colombo, and P. Dellabona. 1994. Immunizing and curative potential of replicating and nonreplicating murine mammary adenocarcinoma cells engineered with interleukin (IL)-2, IL-4, IL-6, IL-7, IL-10, tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor, and gamma-interferon gene or admixed with conventional adjuvants. *Cancer Res.* 54:6022–6026.
 17. Sijts, A.J., F. Ossendorp, E.A. Mengede, P.J. van den Elsen, and C.J. Melief. 1994. Immunodominant mink cell focus-inducing murine leukemia virus (MuLV)-encoded CTL epitope, identified by its MHC class I-binding motif, explains MuLV-type specificity of MCF-directed cytotoxic T lymphocytes. *J. Immunol.* 152:106–116.
 18. Huang, A.Y., P.H. Gulden, A.S. Woods, M.C. Thomas, C.D. Tong, W. Wang, V.H. Engelhard, G. Pasternack, R. Cotter, D. Hunt, et al. 1996. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. *Proc. Natl. Acad. Sci. USA.* 93:9730–9735.
 19. Shortman, K., and Y.J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* 2:151–161.
 20. Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* 2:1098–1100.
 21. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*. 408:740–745.
 22. Dhodapkar, M.V., R.M. Steinman, J. Krasovsky, C. Munz, and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med.* 193:233–238.
 23. Seo, N., S. Hayakawa, M. Takigawa, and Y. Tokura. 2001. Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity. *Immunology*. 103:449–457.
 24. Sica, A., A. Sacconi, B. Bottazzi, N. Polentarutti, A. Vecchi, J. van Damme, and A. Mantovani. 2000. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *J. Immunol.* 164:762–767.
 25. Kaisho, T., O. Takeuchi, T. Kawai, K. Hoshino, and S. Akira. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.* 166:5688–5694.
 26. Sweet, M.J., C.C. Campbell, D.P. Sester, D. Xu, R.C. McDonald, K.J. Stacey, D.A. Hume, and F.Y. Liew. 2002. Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of Toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. *J. Immunol.* 168:392–399.
 27. Zou, W., V. Machelon, A. Coulomb-L'Hermin, J. Borvak, F. Nome, T. Isaeva, S. Wei, R. Krzysiek, I. Durand-Gassel, A. Gordon, et al. 2001. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat. Med.* 7:1339–1346.
 28. Kadowaki, N., S. Ho, S. Antonenko, R. de Waal Malefyt, R.A. Kastelein, F. Bazan, and Y.J. Liu. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Immunol.* 194:863–870.
 29. Weiner, G.J., H.M. Liu, J.E. Wooldridge, C.E. Dahle, and A.M. Krieg. 1997. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. USA.* 94:10833–10837.
 30. Brunner, C., J. Seiderer, A. Schlamp, M. Bidlingmaier, A. Eigler, W. Haimerl, H.A. Lehr, A.M. Krieg, G. Hartmann, and S. Endres. 2000. Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. *J. Immunol.* 165:6278–6286.
 31. Merad, M., T. Sugie, E.G. Engleman, and L. Fong. 2002. In vivo manipulation of dendritic cells to induce therapeutic immunity. *Blood*. 99:1676–1682.
 32. Kawarada, Y., R. Ganss, N. Garbi, T. Sacher, B. Arnold, and G.J. Hammerling. 2001. Nk- and cd8(+) t cell-mediated eradication of established tumors by peritumoral injection of cpg-containing oligodeoxynucleotides. *J. Immunol.* 167:5247–5253.
 33. Ballas, Z.K., A.M. Krieg, T. Warren, W. Rasmussen, H.L. Davis, M. Waldschmidt, and G.J. Weiner. 2001. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. *J. Immunol.* 167:4878–4886.