In vivo Colocalization of Antigen and CpG within Dendritic Cells Is Associated with the Efficacy of Cancer Immunotherapy

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Abstract

Immunostimulatory cytidyl guanosyl (CpG) motifs are of great interest as cancer vaccine adjuvants. They act as potent inducers of Th1 responses, including the activation of cytotoxic CD8+ T lymphocytes (CTL). Whereas animal models have provided clear evidence that CpG enhances antitumor immunity, clinical trials in humans have thus far been less successful. Applying cryosurgery as an instant *in situ* tumor destruction technique, we now show that timing of CpG administration crucially affects colocalization of antigen and CpG within EEA-1+ and LAMP-1+ compartments within dendritic cells *in vivo*. Moreover, antigen/CpG colocalization is directly correlated with antigen cross-presentation, the presence of CTL, and protective antitumor immunity. Thus, failure or success of CpG as a vaccine adjuvant may depend on colocalization of antigen/CpG inside DCs and hence on the timing of CpG administration. These data might aid in the design of future immunotherapeutic strategies for cancer patients. [Cancer Res 2008;68(13):5390–6]

Introduction

Toll-like receptors (TLR) encompass a specialized set of pathogen recognition receptors that are expressed by cells of the innate and adaptive immune system. When triggered by highly conserved danger-associated molecules, such as lipopolysaccharide, certain lipoproteins, and bacterial DNA or RNA, these receptors initiate signaling cascades leading to protective immune responses (1). The identification of natural agonists for TLRs and the development of synthetic equivalents, showing increased stability and half-life *in vivo*, have opened novel therapeutic avenues for the treatment of a large range of infectious diseases and cancer.

In the development of cancer vaccines, the TLR9 agonist cytidyl guanosyl (CpG) oligodeoxynucleotide stands out because of its preferential induction of Th1 responses and tumor-specific cytotoxic CD8+ T lymphocytes (CTL; refs. 2–4). TLR9 is predominantly expressed by B cells and dendritic cells (DC) that internalize and directly respond to CpG (5). On triggering of TLR9, DCs mature and migrate to draining lymph nodes where they present antigens to T and B lymphocytes (6). Importantly, mature DCs acquire the unique ability to present captured antigens on MHC class I molecules, a process known as cross-presentation (7), which is crucial for efficient priming of tumor-specific CTLs (8, 9). As such, CpG administration has been reported to prevent tumor outgrowth in a prophylactic setting and could also eradicate established tumors in mice (10, 11).

Unfortunately, the clinical application of CpG in humans has thus far been less successful (12). A common argument used is the differential TLR9 expression in DCs in mice and man. TLR9 is abundantly expressed in murine and human plasmacytoid DCs (pDC) and in myeloid DCs (mDC) in mice. The expression in human mDCs is, however, less clear as some studies report weak expression (13, 14), whereas a recent study shows that human mDCs contain TLR9 protein in amounts comparable with pDC (15). Moreover, intensive cross-talk between mDCs and pDCs is essential for CpG-induced immune activation in both mice and man (16, 17), indicating a functional link between the DC subsets in both species.

Another explanation for the divergence in the efficacy of CpG application in preclinical and clinical studies is the regimen of CpG administration. We noted that in murine studies, showing the most potent CpG effects, CpG is provided along with the antigen or in close proximity to tumor antigens (2, 18), whereas in clinical studies CpG is administered independent of antigen release (19).

To investigate the importance of timing of TLR agonist administration relative to the availability of tumor antigens *in vivo*, we applied a unique *in situ* tumor destruction model in which the release of tumor antigens can be tightly controlled. Using cryosurgery (20) or radiofrequency ablation of established tumors, we have recently showed that *in situ* tumor destruction, in contrast to tumor resection, leads to the induction of tumor-specific immunity, which can be greatly enhanced by coadministration of CpG (4). In the present study, we show that the failure or success of CpG as a vaccine adjuvant is closely correlated with the colocalization of antigen/CpG within DCs and thus the timing of CpG administration relative to the release of antigen.

Materials and Methods

Mice and cell lines. C57BL/6n mice (6–8 wk old) were purchased from Charles River Wiga and the experiments were performed according to the guidelines for animal care of the Nijmegen Animal Experiments Committee. The B16F10 melanoma cell line was obtained from the American Type Culture Collection and maintained in complete medium [Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal bovine serum (FBS; Greiner Bio One), 100 units/mL penicillin G sodium and 100 μg/mL streptomycin (Pen/Strep), MEM sodium pyruvate (1 mmol/L), NaH2CO3, MEM vitamins, MEM nonessential amino acids (all from Life Technologies), and 20 μmol/L β-mercaptoethanol (β-ME)]. OVA-transfected murine

melanoma cell line B16F10 (B16OVA, clone MO5) was kindly provided by Dr. K. Rock (Department of Pathology, University of Massachusetts Medical Center, Worcester, MA; ref. 21) and cultured in complete medium supplemented with 30 μg/mL hygromycin and 1 mg/mL G418. The D1 cell line, a long-term growth factor–dependent immature splenic DC line derived from B6 mice, was cultured as described before (22). B3Z cells, a T-cell hybridoma specific for SIINFEKL in H-2K^b, which carries a β-galactosidase construct driven by NF-AT elements from the interleukin-2 (IL-2) promotor (23), were cultured in IMDM supplemented with 5% FBS, Pen/Strep, 20 μmol/L β-ME, 0.5 mg/mL hygromycin, and 2 mmol/L L-glutamine. B6-B7.1-sigOVA, a cell line presenting high levels of the MHC class I peptide complex but no OVA class II peptide complexes on its surface (24), was cultured in IMDM, 5% FBS, Pen/Strep, 20 μmol/L β-ME, 0.1 mg/mL hygromycin, and 0.1 mg/mL G418 and used as a positive control.

Tumor model and cryosurgery. Tumors were induced and treated with cryoablation as previously described (4). In challenge experiments, mice were rechallenged with 2×10^4 B16OVA cells 40 d after ablation treatment. Two months later, mice that survived the first tumor rechallenge received a second rechallenge with 5×10^4 B16OVA or B16F10 cells. Mice were sacrificed when tumor volume exceeded 1,500 mm^3.

CpG 1668 (5′-TCCATGACGTTCCTGATGCT-3′) with phosphorothioated backbone (purchased from Sigma-Genosys) was injected in PBS peritumorally (100 μg divided over three injections of 10 μL surrounding the tumor) within 30 min after ablation or 3 or 1 d before or after ablation. We used CpG 1668, which is a type B CpG, similar to the clinical-grade available CpGs currently used in clinical trials.

Enrichment and purification of DCs. Inguinal lymph nodes draining the tumor site were excised and digested in collagenase type II and DNase for 15 min at 37°C. After addition of EDTA and resuspending, cells were filtered and DCs were enriched by positive selection according to the manufacturer's protocol (Miltenyi Biotec B.V.). For confocal experiments, OVA-Alexa488 and CpG-Cy5 double-positive cells were further purified by fluorescence-activated cell sorting (FACS) sorting (Elite cell sorter, Beckman Coulter).

Loading and maturation of DCs. To study the fate of antigen after cryoablation, tumors were injected with fluorescently labeled OVA protein (OVA-Alexa488, 20–40 μg/20 μL). In some studies, Cy5-labeled CpG (CpG-Cy5, 50–100 μg/30 μL) was used to determine the distribution of CpG. Tumor-draining lymph nodes were isolated 48 h after treatment. CD11c-enriched cells were incubated with Fc Block (CD16/CD32; 2.4G2) and stained with anti-CD11c (HL3), biotinylated anti-CD80 (1G10/B7), or isotype controls and streptavidin-phycoerythrin, all obtained from BD Biosciences. Expression of CD80 was analyzed in Alexa$^+$ and Alexa$^-$ cells and gated CD11c$^+$ cells by flow cytometry (FACSCalibur, Becton Dickinson & Co.). As it was reported that uptake of the model antigen OVA by bone marrow–derived DCs largely depended on the mannose receptor (25), we repeated our experiments with bovine serum albumin-Alexa488. No differences in uptake by DCs *in vivo* were observed between the two antigens (data not shown).

Cross-presentation. DCs were enriched from tumor-draining lymph nodes 48 h after ablation and cocultured with B3Z cells for 24 h at 37°C in 96-well plates. The presentation by DCs of SIINFEKL in H-2K^b results in production of β-galactosidase by B3Z cells (23), which can be detected by adding 0.15 mmol/L chlorophenolred-β-D-galactopyranoside (Calbiochem), 9 mmol/L MgCl$_2$, 0.125% NP40, and 100 mmol/L β-ME in PBS. Plates were incubated for 3 h at 37°C and absorbance values were measured using a photo spectrometer (595 nm).

Antigen-specific CTL. Ten days after ablation, spleens and tumor-draining lymph nodes were isolated and single-cell suspensions were prepared. Mixed cell suspensions of lymph node and spleen were plated in 24-well plates in complete IMDM supplemented with human IL-2 (10 units/mL). B16OVA cells were treated overnight with recombinant IFN-γ (50 units/mL), harvested and irradiated (1,600 rad), and added to each well (5×10^4 per well). At days 3 to 4 of culture, cells were harvested and dead cell debris was removed by a Ficoll step. Cells were cultured in fresh 24-well plates for another 4 to 5 d. After a second Ficoll step, cells were stained with FITC-conjugated CD4 (L3T4) or CD8 (53-6.7) and allophycocyanin (APC)-conjugated OVA-K$_b$ tetramers (Sanquin) and analyzed by flow cytometry.

Confocal microscopy. Tumors were injected with OVA-Alexa488 just before cryoablation and CpG-Cy5 was administered peritumorally at the indicated time points. Sixteen hours after ablation, tumor-draining lymph nodes were excised and DCs were enriched by positive selection and OVA-Alexa488 CpG-Cy5 double-positive cells were sorted on a FACS sorting machine. The purified populations were coated on fibronectin-coated or poly-L-lysine–coated glass slides (1.5 h or 15 min), fixed with 2% paraformaldehyde, blocked overnight with PBA, stained with anti-mouse LAMP-1 (eBioscience) or anti-mouse EEA-1 (Abcam) and goat anti-rat/rabbit Alexa568 (Invitrogen), and imbedded in Mowiol (with 1% azide). Images were acquired using a Zeiss LSM510 meta confocal microscope or Bio-Rad MRC1024 operating on a Nikon Optiphot microscope and a Nikon 60× Plan Apo 1.4 immersion lens. Pictures were analyzed with Bio-Rad Lasersharp 2000 and intensity profiles of the merged channel along lines were analyzed using the profile tools of ImageJ software (NIH, Bethesda, MD). Green lines represent the intensity of the antigen marker, whereas the red line represents the intensity of the TLR agonist. Pearson correlation coefficients of total cell images were calculated.

Statistical analyses. Data were analyzed using a two-tailed Student's *t* test or one-way ANOVA for multiple comparisons with Bonferroni as post hoc test. All analyses were performed using GraphPad Software Prism 4.0 for Macintosh.

Results

Timing of CpG administration determines antitumor immunity. To investigate the importance of the presence of CpG
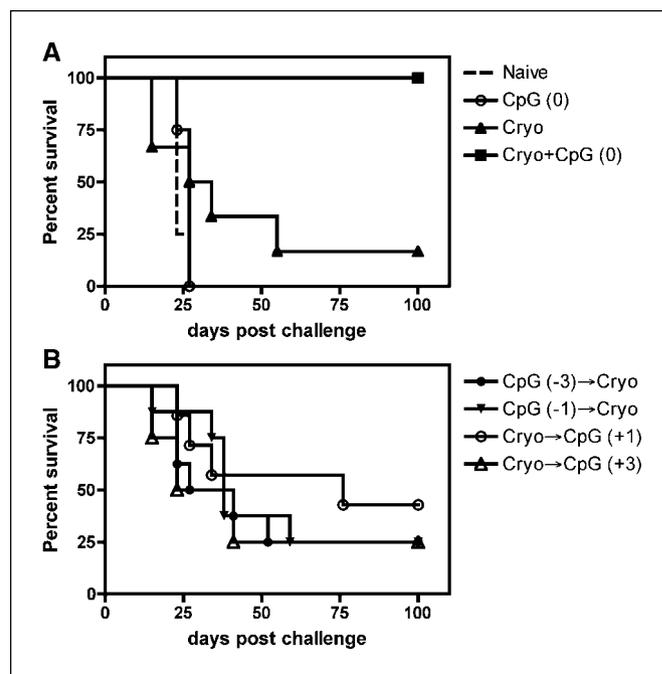

Figure 1. Timing of peritumoral CpG injections and the induction of efficient antitumor immunity. Established B16OVA tumors were treated with cryoablation alone or in combination with peritumoral CpG administrations concurrent with ablation (*Cryo+CpG*) or 1 or 3 d before [*CpG (−3)/CpG (−1)*] or after [*CpG (+1)/CpG (+3)*] ablation. Forty days later, naive and treated tumor-free mice received a s.c. rechallenge with tumor cells (20,000 B16OVA cells) on the contralateral flank. Mice were killed when tumors measured >1,500 mm^3. *A*, survival curves of naive mice versus mice that have been treated with cryoablation alone or in combination with concurrent CpG injections. *B*, in the same experiment, CpG injections were given 1 or 3 d before or after ablation. Data are representative of two independent experiments. Differences between groups presented in *A* (not in *B*) are significantly different (*P* < 0.05) except for naive versus CpG alone. Surviving mice also rejected a second rechallenge with 50,000 B16F10 cells (data not shown), indicating long-term antitumor immunity to B16 antigens other than OVA.

relative to the availability of tumor antigens *in vivo*, we used a recently developed mouse model in which *in situ* tumor destruction by cryoablation provides an instant antigen source for DCs *in vivo*. Hereto, B16OVA tumor-bearing mice were treated with cryoablation and injected with CpG at different time points relative to ablation. Subsequently, tumor-free mice were challenged with a lethal dose of B16OVA cells 40 days after ablation. As reported previously (4), mice treated with cryoablation alone showed prolonged survival, whereas additional CpG injections concomitant with ablation resulted in protective immunity in 100% of the mice (Fig. 1*A*). Remarkably, treatment of mice with CpG injections either 1 or 3 days before or after ablation was much less effective as only 25% to 50% of the mice survived the challenge (Fig. 1*B*).

Analyses of OVA-specific CD8^{+} T cells revealed the superior induction of CTL when CpG was administered immediately after ablation (CpG on day 0: $2.4 \pm 0.6\%$ versus day -3: $0.8 \pm 0.3\%$, day -1: $1.4 \pm 0.7\%$, day $+1$: $0.6 \pm 0.2\%$, and day $+3$: $0.6 \pm 0.4\%$; Fig. 2*A–C*). These data indicate that the timing of CpG administrations relative to antigen availability is decisive for the efficient induction of tumor-specific CTL and subsequent antitumor immunity.

Timing of CpG injection has minor effects on DC maturation and loading with antigen and CpG. To elucidate the underlying mechanisms of protective CTL induction, we studied the quality of DCs following CpG administration in terms of the up-regulation of the costimulatory molecule CD80. To be able to discriminate between antigen[+] and antigen[−] DC in the draining lymph node, tumors were injected with Alexa488-conjugated OVA protein just before tumor destruction. The results show that cryoablation or CpG alone slightly increased CD80 expression on antigen-loaded and unloaded DC, although the expression levels in the latter were markedly lower. When the two treatment modalities were combined, CD80 expression was synergistically enhanced irrespective of the timing of CpG treatment (Fig. 3*A*). These data show that timing of CpG administrations relative to antigen release does not affect the up-regulation of CD80.

DCs were highly successful in collecting both antigen (10–19% of all DCs) and CpG (66–93% of all DCs) in an *in situ* tumor ablation–dependent manner. CpG enhanced the percentages of antigen-loaded DCs but only when administered concurrent with ablation (19%) or after ablation (16%). Injection of CpG before ablation did not have any effect on antigen uptake when compared with cryoablation alone (10%; Supplementary Fig. S1*A*). All antigen-loaded DCs taken from mice receiving any of the combinational treatments also stained positive for CpG, but mean fluorescence levels for CpG were the highest in cells from simultaneously treated
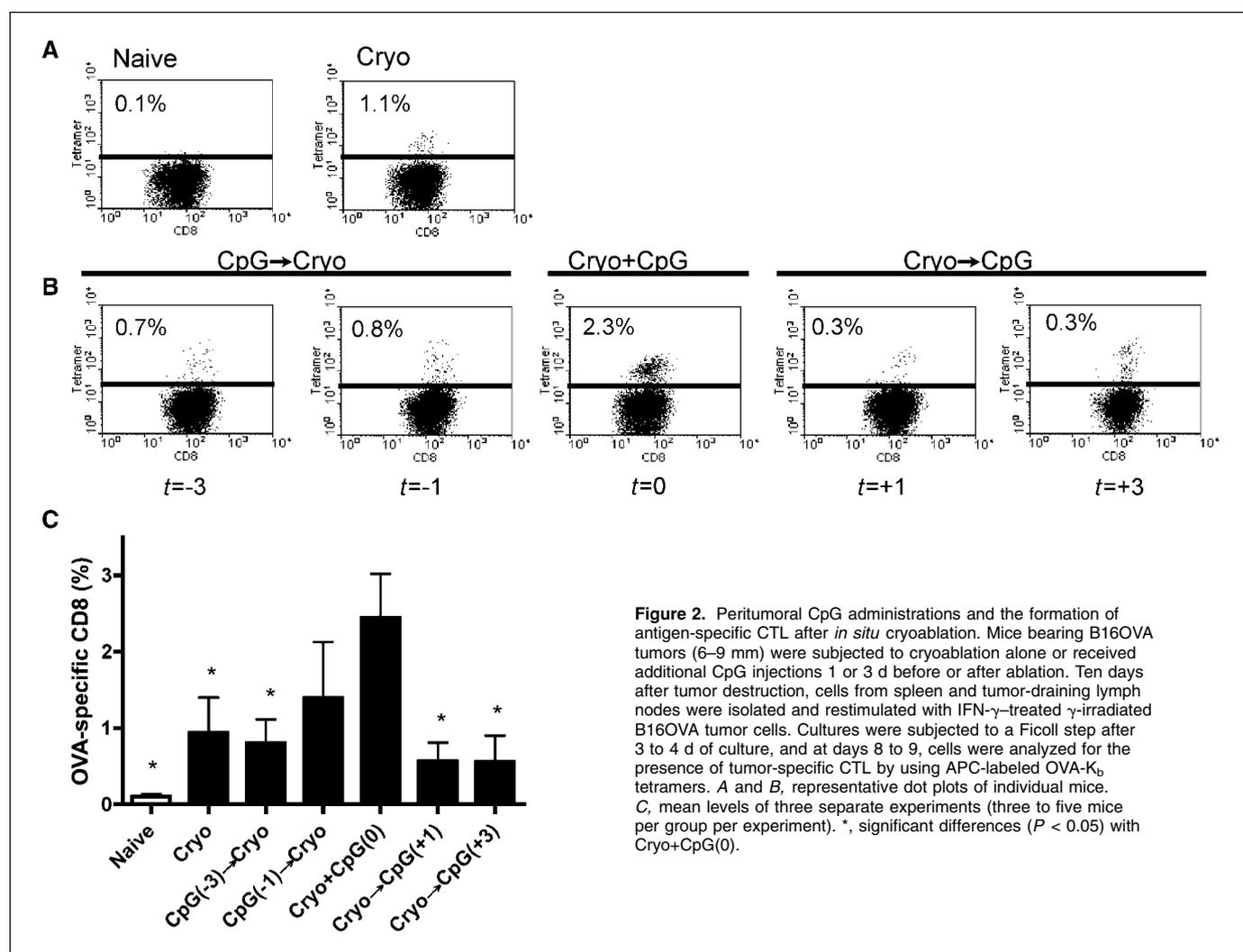

Figure 2. Peritumoral CpG administrations and the formation of antigen-specific CTL after *in situ* cryoablation. Mice bearing B16OVA tumors (6–9 mm) were subjected to cryoablation alone or received additional CpG injections 1 or 3 d before or after ablation. Ten days after tumor destruction, cells from spleen and tumor-draining lymph nodes were isolated and restimulated with IFN-γ–treated γ-irradiated B16OVA tumor cells. Cultures were subjected to a Ficoll step after 3 to 4 d of culture, and at days 8 to 9, cells were analyzed for the presence of tumor-specific CTL by using APC-labeled OVA-K$_b$ tetramers. *A* and *B,* representative dot plots of individual mice. *C,* mean levels of three separate experiments (three to five mice per group per experiment). *, significant differences ($P < 0.05$) with Cryo+CpG(0).
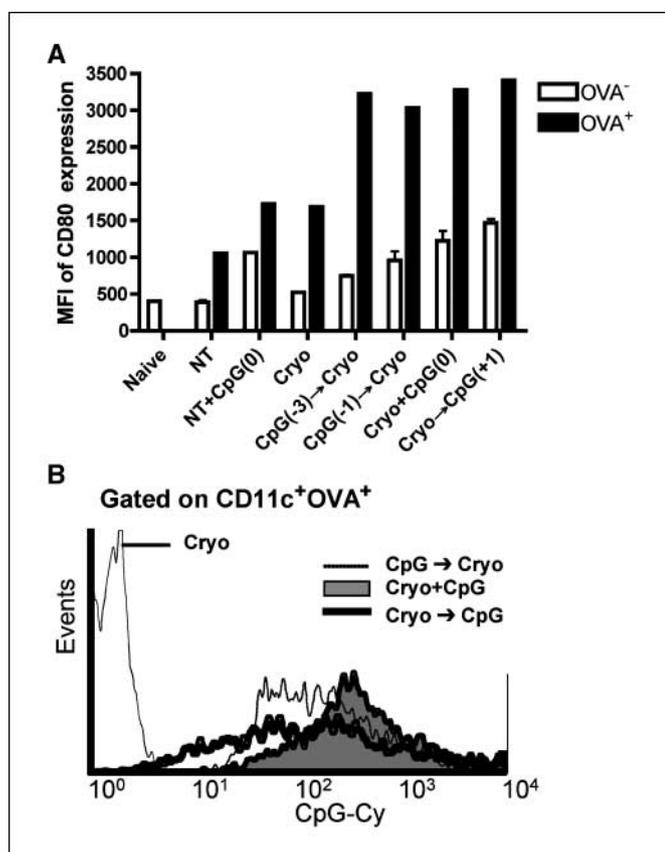
Figure 3. Effects of CpG on *in vivo* maturation and loading of DCs. Established B16OVA tumors (6–9 mm) were injected with OVA-Alexa488 just before ablation to trace the fate of antigen released from the tumor. Tumors were ablated and CpG-Cy5 was injected peritumorally at the indicated time points. CD11c$^+$ DCs were enriched from tumor-draining lymph nodes (four to five mice per group per experiment). *A,* 48 h after treatment, Alexa488$^+$ and Alexa488$^-$ cells were analyzed for the expression of the maturation marker CD80. *B,* mean fluorescence intensity of CpG-Cy5 in Alexa488$^+$ CD11c$^+$ DCs.

mice (Fig. 3*B*; Supplementary Fig. S1*B*). Similar results were obtained in *in vitro* experiments in which all DCs were positive for both CpG and antigen (data not shown).

From these data, we conclude that the differences in the induction of antitumor immunity cannot solely be explained by quantitative differences in antigen/CpG loading or expression of costimulatory molecules by DCs.

Timing of CpG administrations crucially affects cross-presentation. The priming of CTL depends on the unique feature of DCs to present captured antigens on MHC class I, a process known as cross-presentation (26). We next tested the cross-presenting abilities of lymph node DCs directly *ex vivo* on coculture with B3Z cells. As B3Z is a MHC class I–restricted OVA-specific T-cell hybridoma, it is readily activated by K_b-OVA complexes independent of costimulatory molecules or cytokines (23). The data show that DCs from mice treated with cryoablation alone were unable to activate B3Z cells and hence to cross-present antigen (Fig. 4). Remarkably, additional CpG injections day(s) before or after ablation failed to improve cross-presentation by these lymph node DCs. In contrast, DCs isolated from mice injected with CpG at the time of antigen release were able to activate B3Z cells and hence were capable of cross-presentation. To exclude the possibility that a quantitative rather than a qualitative effect was responsible for the differences in cross-presentation, we increased

the numbers of CD11c$^+$ cells in B3Z cocultures. Again, cross-presentation was uniquely induced by lymph node DCs from mice exposed to antigen and CpG at the same time (data not shown). Thus, the ability of DCs to effectively cross-present antigen is highly dependent on the timing of CpG administration.

Cross-priming ability of DCs depends on intra-DC colocalization of antigen and CpG. As these *in vivo* and *in vitro* data imply that DCs that have encountered antigen and TLR agonist at the same time are qualitatively distinct, we determined the fate of antigen and TLR agonist within DCs. As a consequence of cryoablation, tumor cells are opened up and antigenic material, among which soluble antigens, is released (27). To mimic the situation of ablated soluble antigens as much as possible, tumor-bearing mice received an intratumoral injection with OVA-Alexa488 just seconds before ablation and were additionally treated with Cy5-labeled CpG either before or concurrent with tumor destruction.

OVA$^+$CpG$^+$ DCs from tumor-draining lymph nodes were isolated and purified by FACS sorting and then analyzed for the localization of antigen and CpG inside the DCs. Strikingly, lymph node DCs from mice treated with ablation and concurrent CpG injections showed very clear colocalization of antigen and CpG in essentially all DCs (Fig. 5*A*). In contrast, 20% of the DCs derived from mice treated with CpG before ablation showed only partial colocalization, whereas in 80% of the DCs no colocalization was observed (Fig. 5*B*). Recent *in vitro* studies showed that antigen present in EEA-1$^+$ endosomes is efficiently cross-presented, whereas pinocytosed material is directed to LAMP-1$^+$ compartments for presentation to CD4$^+$ T cells (28). We found that colocalization of antigen and CpG was present in EEA-1$^+$ and in LAMP-1$^+$ compartments (Fig. 5*C*), indicating that antigen may be internalized via different endocytic routes.

By calculating the correlation coefficients for antigen/CpG in multiple cells, we observed that the colocalization of antigen/CpG in cells from mice treated with the combinational therapy on day 0 was significantly higher than when the treatments
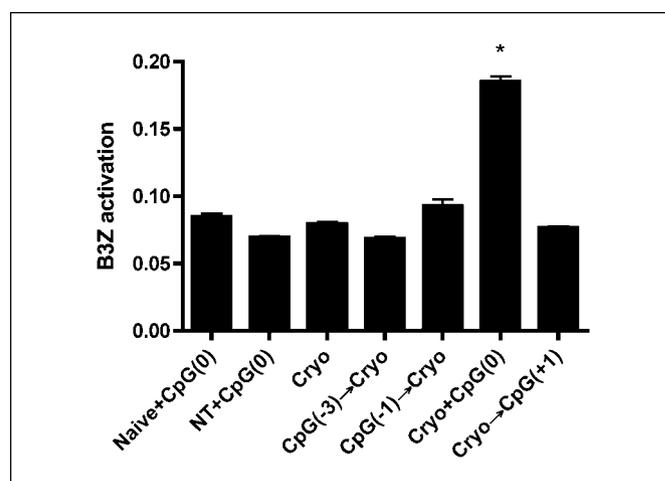

Figure 4. Timing of CpG administration and the induction of cross-presentation *ex vivo* after tumor destruction *in vivo*. Two days after ablation of established B16OVA tumors with or without additional CpG injections at the indicated time points, CD11c$^+$ DCs were enriched from pools of tumor-draining lymph nodes (five mice per group per experiment). Cells were cocultured overnight with B3Z cell and MHC class I–restricted T-cell activation was measured by LacZ production (595 nm). *NT,* tumor-bearing mice that were not subjected to ablation. Timing differences are indicated in days. *, significant differences ($P < 0.05$) compared with all other groups.

were separated in time (Fig. 6). In fact, cells with colocalization from mice treated with CpG before ablation displayed significantly lower correlation coefficients than cells taken from mice treated simultaneously with cryoablation plus CpG. We also compared the CTL responses in mice that were treated with exogenous soluble OVA and CpG at the same time and mice that were treated with CpG before OVA injection. Simultaneous exposure resulted in higher numbers of antigen-specific IFN-γ–producing CTL in spleen and draining lymph nodes when compared with separate exposures in time (data not shown).

These results thus show that colocalization of antigen and CpG in EEA-1 or LAMP-1$^+$ DC compartments *in vivo* highly correlates with the ability of DCs to cross-present antigen to CD8$^+$ T cells resulting in increased protective immunity against tumors.

Discussion

Here, we show that CpG administration synergizes with cryoablation of solid tumors to induce long-lasting antitumor immunity provided that CpG is administered concurrent with
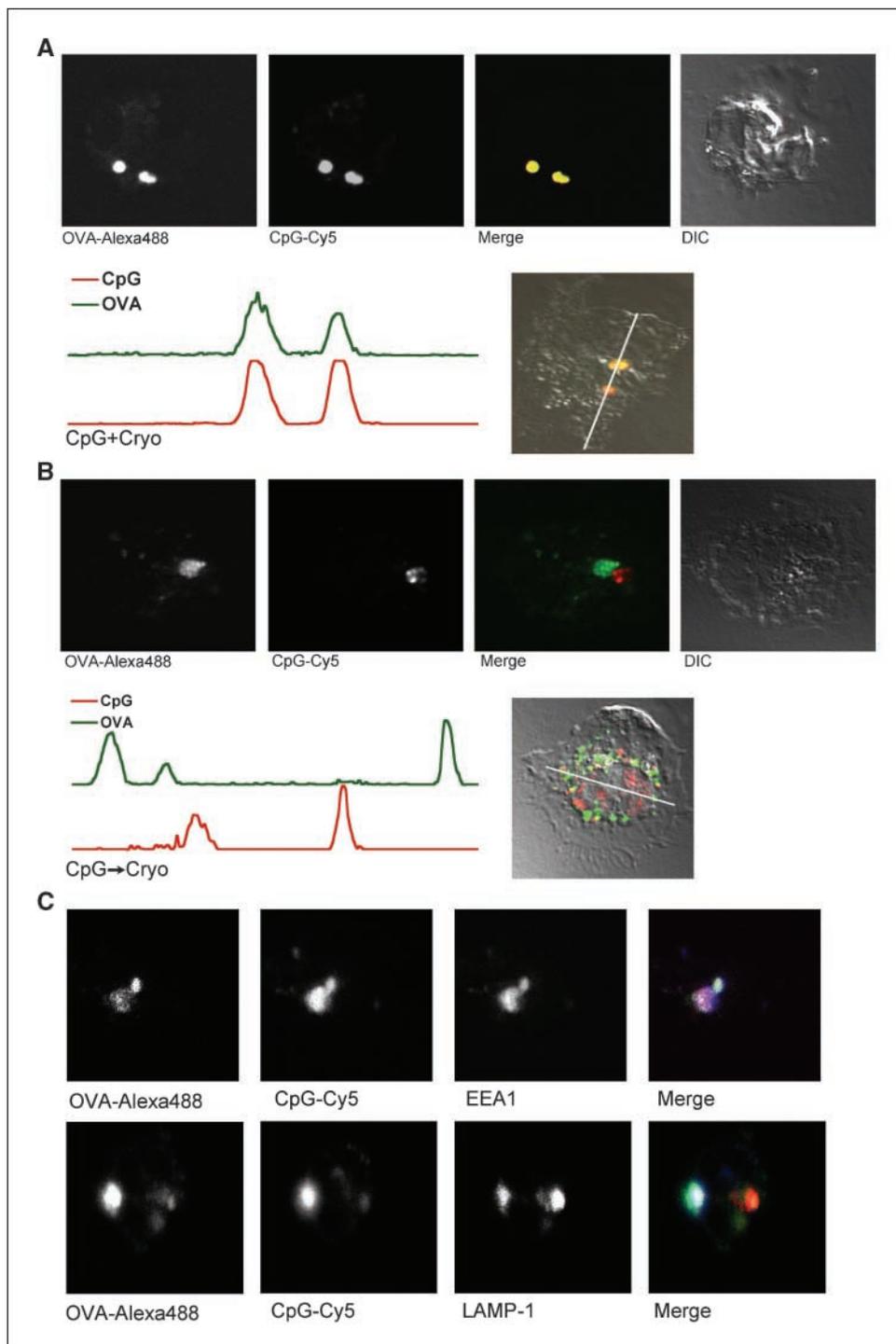

Figure 5. Localization of CpG and antigen within DCs. Established tumors were injected with OVA-Alexa488 just before ablation. Cy5-conjugated CpG was injected concurrent with (*A*) or before (*B*) tumor ablation. Intensity profiles of the merged channel along lines were analyzed using the profile tools of ImageJ software. *Green lines,* intensity of the antigen marker; *red lines,* intensity of CpG. DCs were enriched from tumor-draining lymph nodes 16 h after cryoablation. *C,* the enriched DC fraction (CD11c$^+$) was purified by FACS sorting for double-positive cells for Alexa488 and CpG-Cy5, fixed on fibronectin-coated or poly-L-lysine–coated glass slides, and stained for the endosomal markers EEA-1 or LAMP-1.

ablation. Remarkably, the differences in the efficacy of treatments closely correlate with the colocalization of antigen and CpG within endosomal DC compartments. These data are supportive of the view that colocalization of antigen and CpG within DCs *in vivo* is a prerequisite for DC cross-presentation and the induction of CTL.

Immune stimulation by TLR agonists have been intensively studied but the mechanisms to efficiently enhance tumor-specific CTL responses *in vivo* remain elusive. TLR triggering of DCs programs the expression of costimulatory molecules and cytokines (29) and provides signals that affect the membrane vacuolar system, the cytoskeleton, and the machinery of protein translation and degradation (30). Our data show that the timing of CpG administration does not affect the expression of costimulatory molecules but influences the internalization of antigen and CpG. DCs encountering CpG *in vivo* before ablation internalize less antigen compared with DCs from mice treated with CpG concurrent with or after ablation. These findings are consistent with previous studies showing that TLR triggering first increases the endocytic capacity and then decreases phagocytosis by DCs (31, 32).

Applying fluorescently labeled CpG at different time points *in vivo*, we additionally show that the majority of antigen-loaded DCs are capable of internalizing CpG irrespective of timing. Internalization of antigen and CpG seems, however, not sufficient to induce efficient DC cross-presentation *in vivo* and *in vitro*. Only lymph node DCs isolated from mice treated with CpG concurrent with ablation are able to cross-present antigen to the OVA-specific T-cell hybridoma B3Z. In contrast, CpG administration after ablation also enhances antigen internalization but fails to induce cross-presentation in lymph node DCs. Moreover, *in vitro* experiments in which 100% of the DCs are loaded with antigen confirmed that DCs simultaneously exposed to antigen and CpG are most effective in cross-presentation (data not shown). Together, these data imply that the expression of costimulatory molecules and the level of antigen and/or CpG internalization are not decisive for cross-presentation in this setting.

Recently, Blander and Medzhitov (33) suggested from *in vitro* data that the presence of TLR agonists and antigenic cargo in the same phagosome is crucial for efficient antigen presentation and subsequent priming of CD4+ T cells by DCs. This finding points to a plausible mechanism in which efficient T helper cell induction is dependent not only on the expression of costimulatory molecules and cytokines by DCs but also on phagosome maturation. Whether this also holds true for antigen cross-presentation has been suggested but has never been unequivocally shown. We found that colocalization of antigen and CpG was present in EEA-1+ compartments, which were recently identified to enable antigens to access the cross-presentation pathway *in vitro* (28). Hence, our study provides *in vivo* evidence that the presence of antigen and TLR agonist within the same compartment not only is a pivotal checkpoint for the induction of MHC class II presentation (33) but also allows the antigen present in the same endosomal compartment to gain access to the MHC class I pathway.

Tumor debris after cryoablation contains a complex mixture of numerous different antigens; some will behave as soluble antigens and others will behave as cell-associated antigens. In our studies, we used soluble OVA as a representative marker for soluble antigens that are rapidly released from the tumor following cryoablation. For instance, Huang and colleagues (27) reported a
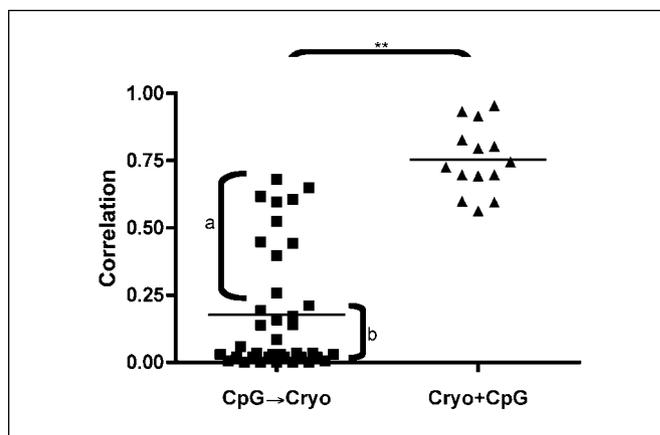

Figure 6. Correlation between CpG and antigen within DCs. Pearson correlation coefficients of total cell images were calculated; the difference between the two groups was highly significant (**, $P < 0.001$). In addition, the mean coefficient of colocalizing cells of CpG→Cryo (*a*) was also significantly different from noncolocalizing cells from the same condition (*b*) and from the mean level after CpG injections concurrent with ablation.

steep rise in serum levels of carcinoembryonic antigen, a cell surface 200-kDa glycoprotein, after cryotherapy in patients with hepatic metastasis from colorectal cancer. Comparable observations were reported by Ghanamah and colleagues (34) after laparoscopic radiofrequency of hepatic metastasis. Whether similar mechanisms are at play in the processing of cell-associated antigens remains to be assessed.

Based on the present results, one would thus predict that TLR-based immunotherapy only occurs in optima forma when DCs internalize antigen and TLR agonist simultaneously. Indeed, antigens covalently linked to TLR agonists induce a stronger immune response than simultaneously but separately administered compounds in mice (35, 36). In addition, intratumoral (2), peritumoral (4, 37), or intralymphatic (18) administrations of CpG *in vivo* were efficient in improving T-cell–mediated immunity, whereas systemic (i.v. or i.p.) application of CpG *in vivo* has been shown to result in T-cell suppression rather than immune activation (38–40). In this context, the timing and location of CpG administration will be an important element in the design of future clinical trials.

We note that the possibility to control the release of antigen by *in situ* tumor ablation as described herein may represent the ideal setting to combine with CpG as this will maximize loading of DCs with both tumor antigens and TLR agonist. As both treatment modalities are already applied in the clinic, it is of great interest to determine the efficacy of CpG in combinational treatment with tumor-ablative strategies in cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Correction: Colocalizing Antigen and CpG Determines Immunity

In the article on how colocalizing antigen and CpG determines immunity in the July 1, 2008 issue of *Cancer Research* (1), the title is incorrect. The title should read "*In vivo* Colocalization of Antigen and CpG within Dendritic Cells Is Associated with the Efficacy of Cancer Immunotherapy".

1. Nierkens S, den Brok MH, Sutmuller RPM, Grauer OM, Bennink E, Morgan ME, Figdor CG, Ruers TJM, Adema GJ. *In vivo* colocalization of antigen and CpG within dendritic cells is associated with the efficacy of cancer immunotherapy. Cancer Res 2008;68:5390–6.

In vivo Colocalization of Antigen and CpG within Dendritic Cells Is Associated with the Efficacy of Cancer Immunotherapy

Stefan Nierkens, Martijn H. den Brok, Roger P.M. Sutmuller, et al.

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