Synergy between *In situ* Cryoablation and TLR9 Stimulation Results in a Highly Effective *In vivo* Dendritic Cell Vaccine

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Abstract

Dendritic cells (DC) are professional antigen-presenting cells that play a pivotal role in the induction of immunity. *Ex vivo*–generated, tumor antigen–loaded mature DC are currently exploited as cancer vaccines in clinical studies. However, antigen loading and maturation of DC directly *in vivo* would greatly facilitate the application of DC-based vaccines. We have previously shown that *in situ* tumor destruction by ablative treatments efficiently delivers antigens for the *in vivo* induction of antitumor immunity. In this article, we show that although 20% of the draining lymph node DCs acquire intratumorally injected model antigens after *in situ* cryoablation, only partial protection against a subsequent tumor rechallenge is observed. However, we also show that a combination treatment of cryoablation plus TLR9 stimulation via CpG-oligodeoxynucleotides is far more effective in the eradication of local and systemic tumors than either treatment modality alone. Analysis of the underlying mechanism revealed that *in situ* tumor ablation synergizes with TLR9 stimulation to induce DC maturation and efficient cross-presentation in tumor-bearing mice, leading to superior DC function *in vivo*. Therefore, *in situ* tumor destruction in combination with CpG-oligodeoxynucleotide administration creates a unique "*in situ* DC vaccine" that is readily applicable in the clinic. (Cancer Res 2006; 66(14): 7285-92)

Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells of the immune system (1). Immature DCs reside in peripheral tissues where they take up and process antigens from their surroundings. In a stimulatory environment, such as in an infection, immature DCs undergo maturation and acquire the capacity to cross-present exogenous antigens in MHC class I (2, 3). In particular, the Toll-like receptor (TLR) proteins initiate the DC maturation process upon recognition of conserved pathogen-associated molecular patterns, like lipopolysaccharide or unmethylated CpG oligodeoxynucleotides (CpG-ODN; refs. 4–6). Maturation is accompanied by migration of the DCs to the draining lymph node, where they subsequently present antigens to immune cells to induce immunity. DCs that did not perceive an activating environment do not mature, and induce tolerance rather than immunity (7).

Because DCs are critical in inducing effective immune responses, *ex vivo*–generated DCs are currently applied to stimulate antitumor immunity in clinical trials (1, 8–11). Although tumor-specific responses have been obtained with tumor antigen–loaded DC-based vaccines, many questions regarding effective tumor antigens and DC migration remain unanswered (8, 12). Moreover, *ex vivo* generation of DC vaccines is time-consuming and costly. *In vivo* loading and maturation of DCs would therefore greatly improve the applicability of DC vaccination.

Recently, an antibody directed against the mouse DC antigen, DEC-205, was shown to target OVA antigens preferentially to DC *in vivo* (13). An alternative approach to create an *in situ* DC vaccine applied retrovirus-mediated expression of the chemokine, CCL20, in tumors to increase the number of intratumoral DCs (14). As previously shown for *ex vivo*–generated DC vaccines (15), both studies confirmed that maturation of *in vivo*–loaded DC by either agonistic anti-CD40 antibodies or the TLR9-ligand CpG-ODN was essential to induce a potent immune response.

Tumor debris left in the body after *in situ* tumor destruction is suggested to be a potential tumor antigen source for DCs *in vivo*, and could provide a direct way of *in situ* DC targeting, without the need for retroviral infection or construction of recombinant proteins. Tumor-ablative treatments, like cryoablation or radiofrequency ablation, have been successfully used in clinical settings to destroy different types of tumors (16–20). Tumor ablation has been associated with the occurrence of immune activation, especially via the induction of inflammatory cytokines (21, 22). Nevertheless, patients treated with an ablative regimen generally develop systemic recurrences as a consequence of the outgrowth of distant micrometastases, implying that, in general, no protective immune response is induced. Indeed, in a new mouse model for *in situ* tumor ablation, we recently showed that only weak antitumor immune responses are induced following tumor ablation alone (23). Adoptive transfer experiments, however, showed that the immunity induced is tumor-specific and T cell–dependent (23).

Here, we show that when the ablation of established B16 tumors (5-7 mm) is combined with CpG-ODN administration, potent antitumor immune responses are induced. Experiments with exogenously injected antigens show that following ablation, a depot is created from which 20% of DCs found in the draining lymph node internalized antigens. Moreover, the data indicate that tumor ablation synergizes with CpG-ODN administration to not only enhance the number and maturation state of lymph node DCs, but also to increase cross-presentation (2), leading to the efficient induction of CD8+ T cells. *In situ* tumor destruction, together with DC activation by CpG-ODN, constitutes a powerful

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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"*in situ* DC vaccine" that is readily applicable in the clinic without prior knowledge of tumor antigens.

Materials and Methods

Animals. Nine- to 11-week-old female C57BL/6n mice were purchased from Charles River Wiga (Sulzfeld, Germany). Animals were held under specified pathogen-free conditions in the Central Animal Laboratory (Nijmegen, the Netherlands). All experiments were done according to the guidelines for animal care of the Nijmegen Animal Experiments Committee.

Tumors. Mice were injected s.c. at the right femur with 500×10^3 cells of the OVA-transfected murine melanoma cell line B16F10 [B16OVA, clone MO5; which was kindly provided by Dr. Kenneth Rock (24)], or wild-type B16F10. Cells were cultured and injected as previously described (23). Tumor volumes were scored every 3 days with the formula: $(A \times B^2) \times 0.4$, in which A is the largest and B is the shortest dimension. Tumors were selected for ablation when their diameters were measured to be between 5 and 7 mm (days 9-10), and only if the tumor was relatively round (>98%).

Cryoablation and *in vivo* procedures. Animals were randomized blindly, properly shaven, and anaesthetized by isoflurane inhalation. The tumor area was disinfected with alcohol and subsequently wetted with distilled water. The tip of the liquid nitrogen cryoablation system (CS76; Frigitronics, Shelton, CT) was placed onto the tumor and after proper freeze attachment, treatment was started. During two treatment cycles of ±70 seconds, the tumor and a small strip around it was frozen to less than −100°C. Treatment was considered successful when the whole tumor appeared frozen macroscopically. In Fig. 1*B*, the ablated tumor was excised, afterwards the skin was seamed by clamps. For antigen monitoring experiments, mice received intratumoral injections of ovalbumin conjugated to Alexa-488 (OVA-Alexa-488; Molecular Probes, Leiden, the Netherlands). Conjugates (20 μg) were injected directly before ablation in 20 μL of PBS. CpG-ODN 1668 (5′-TCCATGACGTTCCTGATGCT-3′; total backbone phosphorothioated; Sigma Genosys, Haverhill, United Kingdom) was used for *in vivo* immune activation. One hundred micrograms of CpG-ODN in 30 μL of PBS was injected 1 hour after ablation, and divided over three injections in the peritumoral area. In Fig. 3*C*, 10×10^6 B16OVA cells were lysed by freeze-thaw cycles and injected s.c. with OVA-Alexa, after which similar ablative treatments were done as described above.

Magnetic bead cell sorting and flow cytometric analysis. For antigen uptake experiments, draining lymph nodes from five to eight mice were pooled, and after crushing, dissociation in DNase/collagenase/EDTA, and passage through nylon mesh (25), cells were counted and sorted by standard MACS isolation with a MACS Midi column. Positive selection of DCs was done using CD11c beads (clone N418; Miltenyi Biotec, Bergisch Gladbach, Germany), whereas negative selection/enrichment was done on the CD90 T cell marker (Thy1.2, 30.H12; Miltenyi Biotec). Sorts were verified by CD3e or CD11c (HL3) staining (data not shown) and cell counts were obtained by manual counting using trypan blue exclusion. Subsequently, cells were stained and analyzed on a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ) with CELLQuest software. Staining was done using the following monoclonal antibodies: CD11c-APC (HL3), CD8b-FITC (53-5.8), CD3e-PE (17-A2), biotinylated CD80 (1G10), biotinylated CD86 (GL-1), and streptavidin-PE. All antibodies were purchased from BD PharMingen (Alphen a/d Rijn, the Netherlands).

Antigen presentation to B3Z and DO11.10. The B3Z T cell hybridoma contains *lacZ* that is induced upon engagement of its TCR that recognizes OVA peptide (257-264) in the context of $H2K^b$ (26), whereas the DO11.10 T cell hybridoma produces interleukin 2 (IL-2) upon engagement of its TCR, which recognizes OVA-peptide (323-339) bound to $I\text{-}A^d$ (but cross-reacts with $I\text{-}A^b$). The β-galactosidase activity of the B3Z T cells (100×10^3/well) after overnight incubation with lymph node–derived, CD11c-sorted DCs (100×10^3/well) was determined by incubating with 0.15 mmol/L of chlorophenol red-β-D-galactopyranoside (Calbiochem, La Jolla, CA), 9 mmol/L of $MgCl_2$, 0.125% Nonidet P40, and 100 mmol/L of β-mercaptoethanol in PBS for 4 hours at 37°C, and OD_{595} was determined. The same procedures were done with the DO11.10 cells, however, IL-2 production was determined using standard ELISA procedures. As controls, the B6MEC transfected with murine B7.1, $H\text{-}2K^b$, and a construct expressing an ER targeting signal sequence, followed by the $OVA_{257-264}$ CTL epitope, SIINFEKL (B6-B7.1-sigOVA), was used (27).

Tetramer stainings. A T cell culture was obtained from spleen and draining lymph nodes of mice 10 days after ablation of a B16OVA tumor or from naïve control mice. Stimulation of these cells (100×10^3) was done by the addition of irradiated, IFN-γ-treated, B16OVA cells (50×10^3) in IL-2 (10 CU/mL) supplemented culture medium. At days 5 and 10, cells were collected and cleaned in a density gradient. At day 10 of culture, cells were stained for 15 minutes at room temperature by OVA-tetramers ($H\text{-}2K^b$) conjugated to allophyrocyanin, which were a kind gift from S.H. van der Burg and counterstained for CD8b.2.

Rechallenge and metastasis model. Forty days after the ablation of B16OVA tumors, mice were challenged by s.c. injection at the contralateral femur of either 15×10^3 B16OVA cells or 15×10^3 EL4 cells (numbers defined by titration). Some mice that rejected the first rechallenge received a second set of rechallenges with 15×10^3 B16OVA cells and 10×10^3 B16F10, inoculated on day 120 (end of Fig. 1*A*) on the right and left flank. Injections were done in 100 μL of PBS. Mice were sacrificed when tumors reached a volume of ±850 mm^3. In the metastasis model, 30×10^3 B16F10 cells were injected contralaterally of the matching tumor to ablate. Three

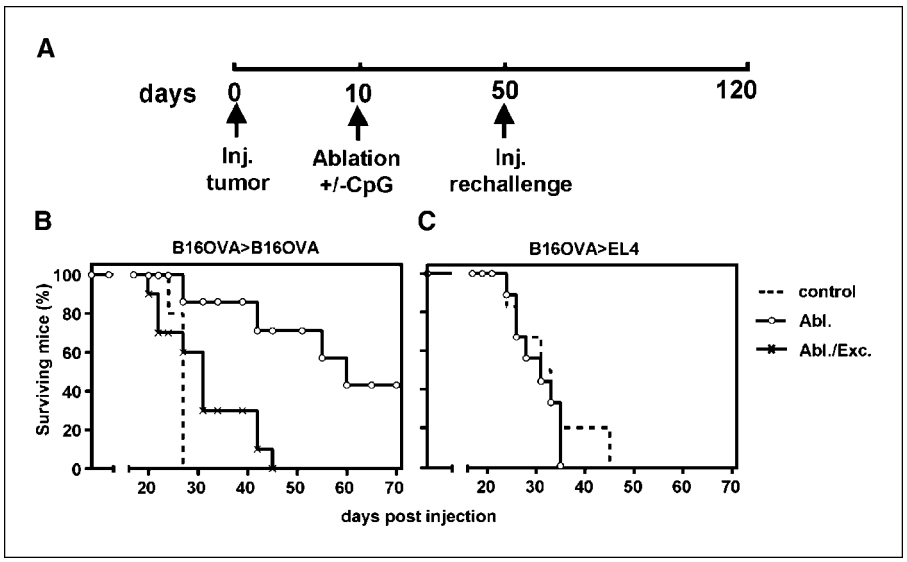

Figure 1. Basal immunity is dependent on depot formation. *A,* time schedule outlining the different treatments as used in the experiments. Ten days after inoculation, the B16OVA melanomas (5-7 mm) were cryoablated. *B* and *C,* directly after ablation, the tumor was excised (-×-) or left *in situ* (-○-). Forty days later, tumor-free mice were rechallenged with B16OVA (*B*) or the nonrelated EL4 tumor (*C*). The survival of mice is depicted in which $T = 0$ corresponds to the time of injection of the tumor rechallenge. As a control, tumor growth was monitored by injection of the same tumor cell dose into naïve mice (- - -). $P < 0.005$ for ablation versus excision. One out of two representative experiments is shown ($n = 5$-10).

days later, cryoablation was done on the primary tumor and 100 μg of CpG-ODN was administered peritumorally. Next, survival from "metastasis" was monitored.

Statistical analysis. All data were analyzed for statistical significance by Student's *t* test, except for the Kaplan-Meier survival curves for which a log-rank test was used.

Results

Cryoablation creates an antigen depot essential for induced antitumor immunity. To determine whether the induction of protective immunity following *in situ* tumor destruction was dependent on the presence of the antigen depot, established B16OVA tumors (5-7 mm) were cryoablated. Successfully ablated mice (70% remained recurrence-free) were rechallenged with either B16OVA or nonrelated EL4 thymoma cells according to the time schedule in Fig. 1*A*. Ablated tumors were excised directly following ablation. In line with previous data,[3] cryoablation of B16OVA tumors resulted in partial protection against a lethal B16OVA rechallenge (Fig. 1*B*), but had no effect on the outgrowth of EL4 tumors (Fig. 1*C*). Excision of cryoablated B16OVA tumors directly after ablation completely abrogated the observed antitumor effect (Fig. 1*B*). These data show that the tumor-specific immune response induced following cryoablation is critically dependent on the presence of the ablated tumor material.

Enhanced antitumor immunity upon ablation with CpG-ODN administration. The results from Fig. 1 also emphasize that the immunity developed against tumor rechallenges is suboptimal. To explore whether the TLR9 ligand, CpG-ODN 1668, could result in the enhancement of antitumor response, CpG-ODN was administered as a single peritumoral injection directly following ablation. We observed that CpG-ODN injection alone, without ablation, did not have any effect on either the outgrowth of the primary tumor (Fig. 2*C*) nor lethal tumor rechallenges 40 days later (Fig. 2*D*). Interestingly, the combination of ablation of the tumor plus CpG-ODN administration resulted in complete protection against the B16OVA rechallenge 40 days later (Fig. 2*A*). Thus, these data show that the combination of *in situ* tumor destruction and CpG-ODN administration is superior in inducing antitumor immunity relative to either treatment alone.

To study immunity to different antigens than the immunodominant OVA protein, the protected mice from Fig. 2*A* received a second set of rechallenges, including wild-type B16F10 tumors. These mice completely rejected the rechallenge with B16OVA (data not shown) but, more interestingly, were also partly protected against the poorly immunogenic, parental B16F10 tumor cells (Fig. 2*B*). Importantly, this occurred only when mice received cotreatment with CpG-ODN. This suggests that the combination treatment induces a potent memory response which is not only directed against the immunodominant epitopes but also to other (unknown) antigens expressed by the parental tumor.

Cryoablation leads to efficient *in vivo* antigen acquisition by lymph node CD11c(+) DC and synergizes with CpG-ODN in *in vivo* DC maturation. As we have recently found that following tumor ablation antigens are almost solely present in the CD11c(+) fraction at 1 day and even 3 days after ablation,[3] we set out to explore the effect of *in situ* tumor destruction plus CpG-ODN administration on DCs. These effects could provide a possible

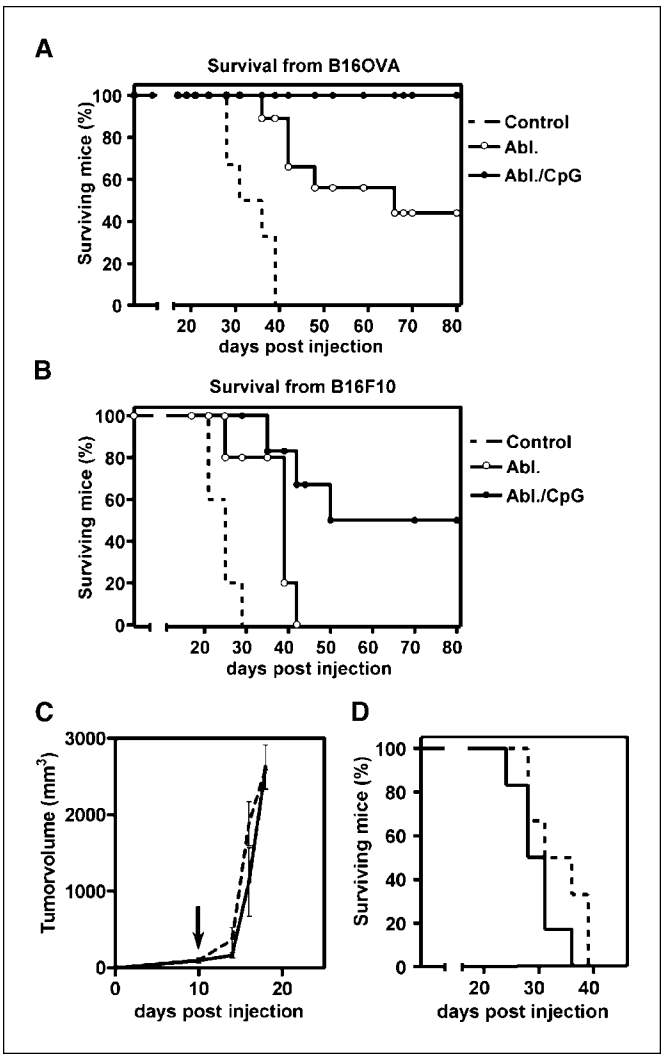

Figure 2. Potent antitumor immunity following combined ablation/CpG-ODN treatment. Mice with established B16OVA melanomas (5-7 mm) were cryoablated. One hour after ablation, mice received 100 μg of CpG-ODN (●) or PBS (○) peritumorally. Forty days later, a rechallenge with 15×10^3 B16OVA cells was given s.c. in the contralateral leg. *A,* survival curve showing complete protection of tumor outgrowth after ablation with CpG-ODN. $P < 0.005$ for all combinations. One out of three representative experiments is shown (n = 5-11). *B,* survival curve of tumor-free mice from (*A*), after secondary rechallenges. Following rejection of the primary B16OVA rechallenge in (*A*), surviving mice were rechallenged with 10×10^3 wild-type B16F10 cells inoculated s.c. on the left flank, and simultaneously with 15×10^3 B16OVA cells on the right flank. Depicted is survival from the B16F10 tumors (n = 5-6 per group). $P < 0.05$ for all combinations. All B16OVA tumors were rejected again (data not shown). As a control, tumor growth was monitored by injection of the same tumor dose into naïve mice (- - -). $T = 0$ corresponds to the time of injection of the tumor rechallenge. One out of two representative experiments is shown. *C* and *D,* control experiment showing that CpG-ODN by itself is insufficient to eradicate the primary tumor or rechallenges. Established B16OVA tumors (5-7 mm) were injected with 100 μg CpG-ODN (i and ↓) or PBS (- - -). Tumor growth was monitored in time (*C*). S.c. injection of 100 μg of CpG-ODN (i) or PBS (- - -), 40 days prior to a B16OVA challenge (15×10^3 cells) did not affect survival of the mice (*D*).

mechanism responsible for the potent induction of antitumor immunity. In order to visualize the uptake of antigens by flow cytometry, chicken egg ovalbumin conjugated to the fluorophore Alexa-488 (OVA-Alexa) was injected intratumorally prior to ablation. As shown in Fig. 3*A* and Supplementary Fig. S1, >20% of all CD11c(+) DC became OVA-Alexa(+) after cryoablation, whereas much less antigen uptake was observed in nonablated

[3] Unpublished results.

mice (<5%). Coadministration of CpG-ODN did not significantly affect the loading of DCs in the draining lymph node. Confocal microscopy showed that these antigens were indeed internalized and not just sticking to cell membranes (data not shown).

Next, we studied the effect of cryoablation and CpG-ODN on DC maturation. Thus, OVA-Alexa(+) or OVA-Alexa(−) DCs were analyzed for expression of the maturation markers CD80 and CD86. Phenotypical analysis of OVA-Alexa(+) DC showed a 3-fold increase in CD80 expression relative to OVA-Alexa(−) DC in tumor-bearing and naïve mice [mean fluorescence intensities (MFI), 1,007, 332, and 315, respectively, Fig. 3*B*; Supplementary Fig. S2]. Following ablation, CD80 expression further increased on OVA-Alexa(+) DCs but had no effect on OVA-Alexa(−) DC (MFI, 1,374 and 369, respectively). This finding indicates that DC which acquired OVA-Alexa, preferentially up-regulate CD80, which is further enhanced by cryoablation.

CpG-ODN injection alone also resulted in a significant increase in CD80 expression on OVA-Alexa(−) DC in both control and tumor-bearing mice (MFI 315 to 536 and 332 to 605) and in a further increase on OVA-Alexa(+) DC from tumor-bearing mice (MFI 1,007 to 1,342; Fig. 3*B*). When CpG-ODN injection was combined with ablation, an additive maturation of OVA-Alexa(−) DC (MFI 369 to 999) was observed. Most surprisingly, CD80 expression on OVA-Alexa(+) DC increased tremendously relative to the increase observed in tumor-bearing mice (MFI 1,374 to 2,682 relative to MFI 1,007 to 1,342). This effect on DC maturation of OVA-Alexa(+) DC is synergistic when compared with the effects seen when ablation or CpG-ODN are provided separately. Moreover, the observed synergy was not related to the eradication of the tumor and its immune-suppressive environment, as *in vivo* ablation of *ex vivo*–created tumor lysates showed comparable synergy on maturation (Fig. 3*C*). Similar, but somewhat less profound, effects were observed for CD86 expression (data not shown).

Increased DC numbers in draining lymph nodes after cryoablation plus CpG-ODN administration. Analysis of the absolute numbers of CD11c(+) DC in the draining lymph nodes following the different treatments showed that both injection of only CpG-ODN or the presence of a tumor resulted in a 2-fold increase in DC numbers relative to naïve mice (Fig. 3*D*). Ablation alone resulted in a 3-fold increase in the number of lymph node DCs, whereas tumor ablation combined with CpG-ODN treatment was again most effective. This implies that the total number of DCs actually loaded with tumor antigen is even higher than can be concluded from the relative numbers.

CpG-ODN following ablation enables cross-presentation of tumor derived antigens. To assess the function of DCs following ablation, CpG-ODN administration, or the combination treatment,

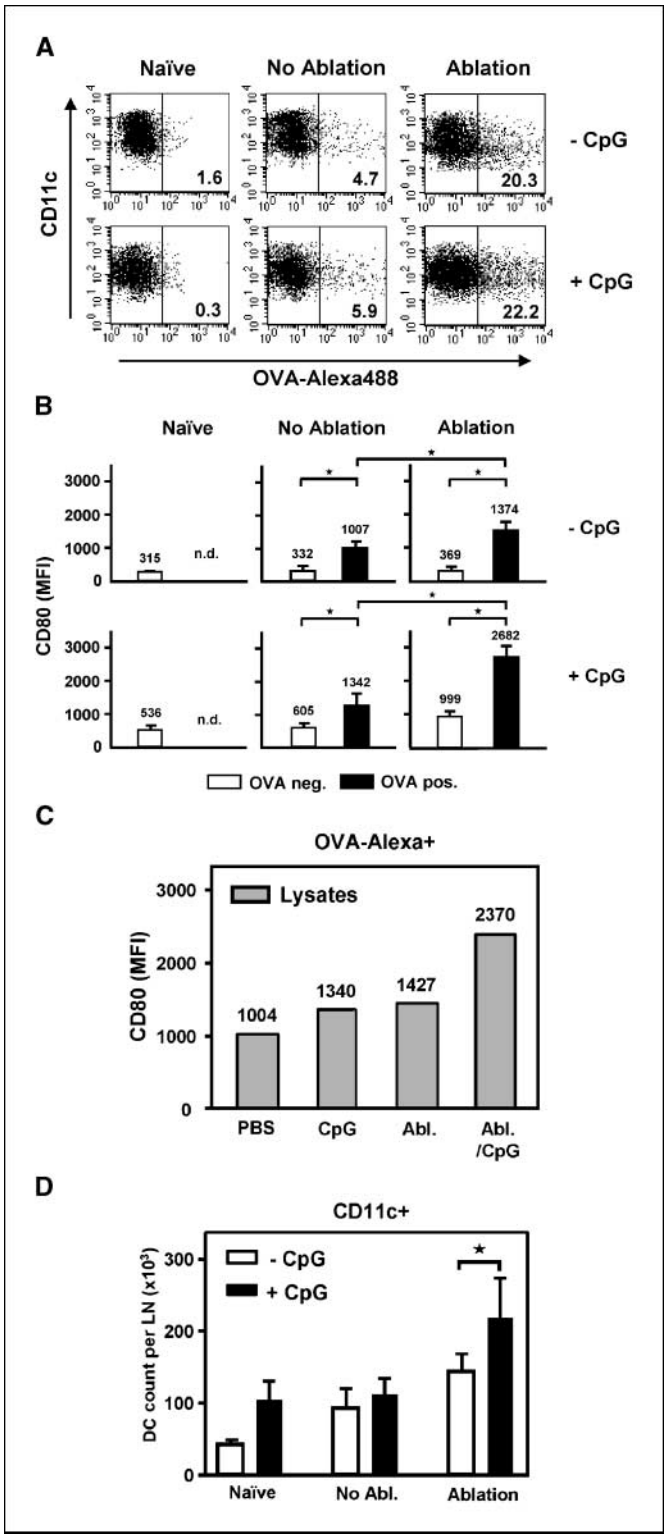

Figure 3. OVA-Alexa-488 uptake and synergistic DC maturation following combined ablation/CpG-ODN treatment. *A* and *B,* fluorescence-activated cell sorting analysis of CD11c(+) DCs isolated from pooled lymph node suspensions of naïve, tumor-bearing, or tumor-ablated mice ($n = 6$ per group). Mice received 20 μg of ovalbumin conjugated to Alexa-488 (OVA-Alexa-488) intratumorally just prior to the time point of ablation. One hour later, 100 μg CpG-ODN was injected peritumorally as indicated. Two days after the indicated treatments, the number of OVA-Alexa(−) and OVA-Alexa(+) DCs was determined (*A*; Supplementary Fig. S1) and separately analyzed for CD80 expression (*B*). *A, values,* percentages of OVA-Alexa(+) cells within the CD11c(+) fraction. *B, columns,* MFI from three independent experiments; *bars,* SD (*, $P < 0.05$). A set of the corresponding histograms is provided in Supplementary Fig. S2. *C,* control experiment showing that DC maturation is ablation-dependent. 10×10^6 B16OVA cells were lysed by freeze-thaw cycles and injected s.c. After coinjection of OVA-Alexa and CpG-ODN as indicated, the injection depot was cryoablated or left untreated. Next, CD80 expression on Alexa-488 positive DCs (six mice pooled per group) was determined. MFI's obtained from one of two experiments with comparable results. *D,* effect of the indicated treatments on total number of CD11c(+) cell counts per lymph node. Two days after the indicated treatments, pooled lymph node suspensions were subjected to magnetic bead sorting on CD11c. Total cell counts of the obtained DC fractions, corrected for the number of lymph nodes are depicted in the graph. *Columns,* mean obtained from three independent experiments; *bars,* SD (*, $P < 0.05$).

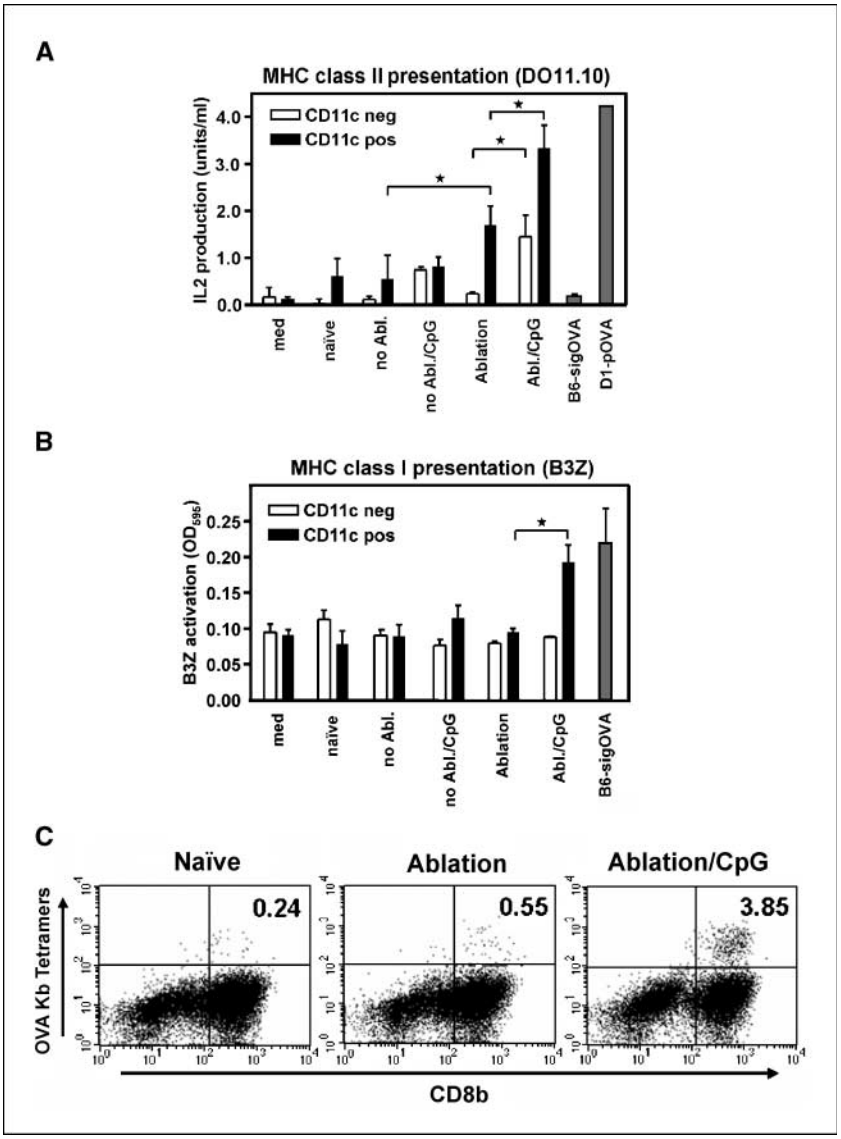

Figure 4. Efficient cross-presentation following combined ablation/CpG-ODN treatment. *A* and *B,* 2 days after ablation of B16OVA tumors with or without coinjection of 100 μg of CpG-ODN, CD11(+) DCs were sorted from draining lymph nodes (n = 7-8). Subsequently, they were cultured overnight with MHC class I or MHC class II, OVA-specific T cell hybridomas (B3Z and DO11.10, respectively). T cell activation was measured by IL-2 production (*DO11.10*; *A*) or LacZ production (*B3Z*; *B*). B6-sigOVA is a cell line presenting high levels of the MHC class I OVA peptide complex but no OVA class II peptide complexes on its cell surface. "med" is a medium control with only T cells. *Columns,* means from triplicates; bars, SD (*, $P < 0.05$). One experiment out of two is shown. *C,* induction of OVA-specific CTL was determined. At day 10 after ablation, a mix of lymph node and spleen cells was obtained from mice treated as indicated. T cells were harvested and restimulated with irradiated, IFNγ-treated B16OVA cells, and IL-2 for 7 days, followed by staining with OVA tetramers (K^b) and anti-CD8b. Depicted numbers represent the percentages of tetramer-positive cells within the CD8b+ population.

we determined the ability of the CD11c(+) DC to cross-present antigens to MHC class I- and II–restricted OVA-specific T cell hybridomas. Significant activation of MHC class II–restricted DO11.10 cells was readily observed upon coculturing with CD11c(+) DCs isolated from tumor-ablated mice (Fig. 4*A*). In contrast, CD11c(+) DCs from tumor-bearing mice without ablation did not activate DO11.10, nor did CD11c(−) cells. The administration of CpG-ODN to these mice increased DO11.10 activation only to a small extent. CpG-ODN administration after ablation significantly increased DO11.10 activation by CD11c(+) cells compared with tumor ablation or tumor alone, and now the CD11c(−) cell fraction of these mice also displayed DO11.10-activating properties.

Next, we analyzed the cross-presenting capacity of these DCs by testing their ability to activate the MHC class I–restricted OVA-specific B3Z T cells. Strikingly, only DCs from mice treated with cryoablation plus CpG-ODN were able to efficiently activate B3Z T cells (Fig. 4*B*). Little or no activation of B3Z T cells could be discerned after ablation or CpG-ODN administration alone. Because DCs from ablated mice that did not receive CpG-ODN treatment processed and presented antigens to the MHC class II–restricted DO11.10 T cells, these data show that efficient cross-presentation after ablation by DCs *in vivo* is codependent on CpG-ODN.

Finally, we analyzed mice with ablated B16OVA tumors (in the absence of exogenously added OVA) for the presence of OVA-specific CD8+ T cells by K^b-tetramer analysis. The data revealed that, consistent with the observed cross-presentation, abundant OVA-specific CD8+ T cells were only present in combined ablation/CpG-ODN treatment (Fig. 4*C*).

Collectively, these data thus indicate that cryoablation plus CpG-ODN results in synergistically improved DC functions of OVA-Alexa(+) DC, as well as a large increase in the total number of these DCs.

CpG-ODN injection following ablation generates therapeutic antitumor immunity. Because ablation is often used in advanced disease, when micrometastases are present, the potency of the model was analyzed in a stringent wild-type B16F10 metastasis model. Thus, B16F10 tumor-bearing mice were injected with 30×10^3 B16F10 cells in the contralateral flank

3 days prior to the ablation/CpG-ODN treatment of the primary B16F10 tumor, to mimic the presence of a second tumor. Subsequently, the growth of this second tumor (metastasis) was monitored. Interestingly, next to successful elimination of the primary tumor in mice treated with ablation/CpG-ODN, regression of the established contralateral B16F10 metastasis was observed in 40% of the mice, along with a growth reduction in the others. As expected, cryoablation or CpG-ODN alone had little or no effect on the outgrowth of metastasis and resulted in 100% tumor take (Fig. 5*A*).

In the course of the experiments described above, we made the intriguing observation that CpG-ODN administration improved the success rate of the B16OVA cryoablation itself. Whereas 30% of the mice normally developed a local recurrence within 15 days following cryoablation, ablation plus CpG-ODN treatment completely prevented the outgrowth of local recurrences (Fig. 5*B*).

Thus, these results show that ablation directly followed by CpG-ODN administration has a fast local effect in addition to the induction of a broad protective memory response.

In conclusion, these data indicate that *in situ* tumor destruction plus TLR9 stimulation leads to a more potent local and systemic antitumor response than either treatment modality alone. This treatment regimen allows for direct antigen-loading and maturation of DC *in vivo* without the delivery of defined tumor antigens.

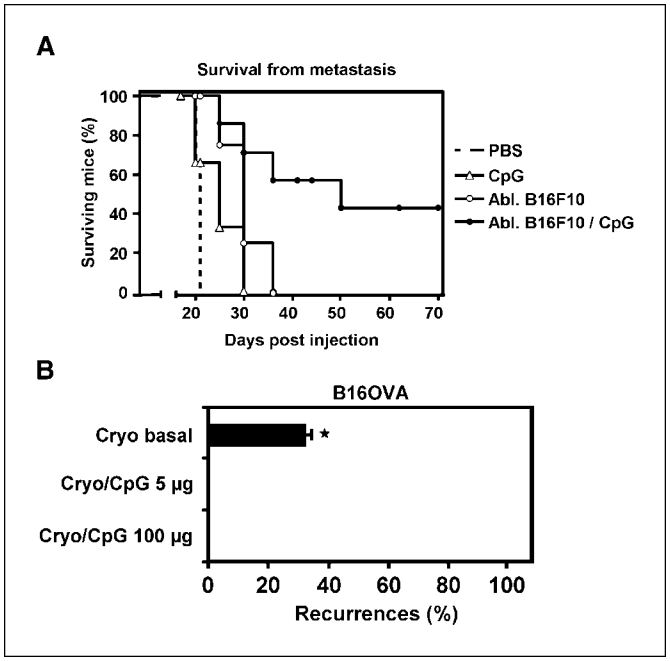

Figure 5. CpG-ODN improves therapeutic outcome of ablation. *A,* stringent wild-type B16 metastasis model. Three days before ablation of a wild-type B16F10 tumor with (●) or without (○) coadministration of 100 μg CpG-ODN, a second dose of 30×10^3 B16F10 cells (metastasis) was inoculated contralaterally of the primary tumor. After successful ablation, survival of the mice from metastasis was monitored. Survival was compared with an injection of the same tumor dose into naïve mice without primary tumor (- - -). S.c. injection of CpG-ODN contralateral of the metastasis was done as a control (△). $T = 0$ corresponds to the time of injection of the metastasis. $P < 0.005$ for ablation/CpG versus ablation alone. One out of two representative experiments is shown (n = 5-12 per group). *B,* after ablation of B16OVA tumors, mice were monitored for tumor recurrence at the site of ablation. Mice that received only ablation or ablation with 5 or 100 μg CpG-ODN peritumorally are shown. *Column,* means obtained from five independent experiments; *bars,* SD (*, $P < 0.05$; n = 20-60).

Discussion

In recent years, *ex vivo*–generated mature DC have been shown to evoke tumor-specific responses in patients with cancer (28–30). DC vaccination is, however, time-consuming and expensive, and in many cases, the antitumor response falls short in strength to cure patients with established tumors. Herein, we report that tumor debris created by the ablation of established tumors comprises an effective antigen source for DC *in vivo*. Moreover, we show that tumor ablation synergizes with the TLR9-ligand CpG-ODN to augment DC function and cross-priming *in vivo*. This creates an effective *in situ* DC vaccine capable of inducing protection against lethal tumor rechallenges as well as regression of preexisting metastases.

In situ tumor destruction with cryosurgery, radiofrequency, or laser ablation has received increasing attention as a treatment modality for focal cancer (16–20). However, little is known regarding the induction of immune responses after *in situ* tumor destruction, or the fate of tumor debris. Applying a mouse B16 tumor model for cryoablation, we recently showed that tumor debris remaining *in situ* after tumor destruction creates an effective antigen depot for DCs *in vivo*. Using intratumoral injection of ^{111}indium-labeled keyhole limpet hemocyanin proteins prior to ablation, we could monitor the fate of tumor debris and show that tumor-derived antigens were efficiently taken to the draining lymph node. We showed that within the draining lymph node, up to 20% of DCs contained antigen as soon as 1 day and for at least 3 days following ablation.[4] The current analysis of the immune response induced following tumor ablation revealed that removal of the antigen depot directly after ablation completely abrogated the development of specific immunity. Although a tumor-specific immune response could be observed when leaving the tumor debris *in situ*, mice were poorly protected against a subsequent tumor challenge. These data indicate that despite the efficient loading of DCs which takes place in mice, immune activation following tumor ablation is suboptimal.

It is currently not known whether antigens are taken up by DCs migrating into the tumor tissue, and subsequently, to the draining lymph nodes, or that antigen is floating passively towards the draining lymph nodes where lymph node–residing DCs take it up. A recent study showed that lymph node DCs accumulated antigen deposited in s.c. tissue (34). It showed that after injection, antigen was first detected in lymph node–residing DCs, followed by a second wave of antigen-positive DCs that migrated from the periphery into the lymph node. Both waves were required for efficient immune response induction and were dependent on the presence of the challenge site. Our finding that both antigen-loaded DCs could be discerned from the lymph node at days 1 and 3 after ablation suggests that similar dynamics take place following *in situ* tumor ablation.

In order to increase antitumor immunity following ablation, we explored the stimulation of TLR9 by CpG-ODN either alone or in combination with ablation. Peritumoral injection of CpG-ODN has previously been shown to elicit coordinated T cell responses and induction of antitumor immunity in relatively immunogenic mouse models (35). In more challenging settings with large tumor masses and less immunogenic tumors, the efficacy of CpG-ODN alone was less profound. Using different tumor models, we now show that

[4] Unpublished results.

ablation plus CpG-ODN treatment of 5 to 7 mm B16OVA tumors resulted in complete protection against a lethal B16OVA tumor rechallenge, and coincided with the presence of OVA-specific CD8+ T cells. As neither CpG-ODN, nor ablation alone was able to protect all mice, we conclude that both CpG-ODN and ablation are essential in the induction of immunity. Recent mouse studies also indicated that CpG-ODN induced tumor growth delay and improved survival when combined with radiotherapy (36) or chemotherapy (37–40), although the mechanism responsible for these effects remain to be determined.

Interestingly, the combination treatment of B16OVA tumors was also able to protect 50% of the mice against outgrowth of the poorly immunogenic parental B16F10 tumor, indicating the simultaneous induction of responses against multiple epitopes. Moreover, local CpG-ODN stimulation also improved the efficacy of the ablation itself, most likely by activation of the innate immune system. When applied to a wild-type B16F10 metastasis model, ablation plus CpG-ODN treatment induced significant regression of pre-existing B16F10 metastases (Fig. 5*A*). In addition, we also made the striking observation during the course of our studies that local recurrence percentages at the ablated site were reduced from 30% to 0% when CpG-ODN was administered following ablation (Fig. 5*B*).

To unravel the mechanism responsible for the induction of potent antitumor immunity following combination treatment *in vivo*, we focused on the effects on DC. Analysis of the maturation state of antigen-loaded and nonloaded DC in naïve, tumor-bearing, tumor-ablated, and CpG-ODN-treated mice revealed several interesting phenomena. First, DCs that contained antigen expressed significantly higher levels of costimulatory molecules than antigen-negative DC. These data are in line with *in vitro* data indicating that antigen uptake could affect DC activation (41). Secondly, ablation resulted in a significant further increase in costimulatory molecule expression on antigen-positive but not antigen-negative DCs. Thirdly, CpG-ODN administration alone resulted in an increase in costimulatory molecule expression on antigen-positive DCs, but also on antigen-negative DCs. Importantly, the observed increase in the number and maturation state of antigen-loaded DCs, induced by CpG-ODN or ablation alone, is apparently not sufficient to induce complete tumor protection of mice.

The finding that antigen uptake–dependent DC maturation was comparable with or without exogenously added OVA-Alexa (see Supplementary Fig. S3) provides strong support for these model antigens truly mimicking endogenous tumor antigens. In addition to this, OVA-specific T cells were readily observed following ablation plus CpG-ODN administration in a setting in which no additional OVA was administered (Fig. 4*C*). We also note that neither antibodies used during positive MACS sorting, nor TLR-ligands often present in OVA batches (42) biased the CD80/CD86 staining on DCs, as DCs purified by negative selection showed similar results and OVA-Alexa did not mature DCs *in vitro* (data not shown; Supplementary Fig. S4).

Strikingly, CpG-ODN administration in combination with tumor ablation resulted in a tremendous increase in costimulatory molecule expression (Fig. 3*B*). These data are indicative of a synergistic interplay between signals induced by ablation and those resulting from TLR9 triggering. The exact nature of these ablation-dependent signals needs further clarification, but may well represent cytokines released after ablation (43–45). For instance, it has recently been shown in a human study that tumor necrosis factor-α and IL-1β levels were markedly enhanced following radiofrequency ablation or ablation by ethanol instillation (46). Alternatively, other endogenous mediators might be involved in the synergistic maturation of DCs observed (47–49). Upon *in situ* tumor destruction, endogenous danger signals like heat shock proteins or acute phase proteins are readily released from the damaged tissue/tumor, which may contribute to immune activation. For instance, it was found that heat shock protein expression at the tumor margins was increased when tumors were ablated by laser ablation (50). An additional explanation for the synergistic DC maturation could be the destruction of the tumor itself. Ablation of the tumor would eliminate tumor-induced factors preventing full responsiveness to CpG-ODN (15). However, because synergistic DC maturation was comparable when ablation with CpG-ODN was done on intact tumors or on injected B16OVA tumor lysates, in our model, the negative influence of the tumor milieu on DC function is limited (Fig. 3*C*).

Combined ablation and CpG-ODN treatment not only affected the phenotype of lymph node DCs but also their capacity to cross-present antigens and activate T cells. Although ablation alone was able to generate DCs that presented antigens in MHC class II, CpG-ODN coadministration was essential to obtain MHC class I presentation and an increase in tetramer(+) T cell numbers. Figure 4*A* shows that upon CpG-ODN administration, MHC class II presentation could also be observed in the CD11c(−) fraction. This could possibly be caused by increased numbers of antigen-presenting B cells or pDC in this fraction, however, this has not been investigated. The role of CpG-ODN in cross-presentation by DCs *in vitro* has recently been reported (51, 52). Our data now provide direct evidence for the crucial role of CpG-ODN in the cross-presentation of antigens by DCs *in vivo*.

Alternative approaches to target antigen to DCs, such as by making use of the DC antigen, DEC-205 (13), or by retrovirus-mediated expression of the chemokine, CCL20, in tumors (14), confirmed that maturation of *in vivo*–loaded DCs by either agonistic anti-CD40 antibodies or the TLR9 ligand CpG-ODN was essential to induce a potent immune response. Our results showed that the tumor debris left in the body after *in situ* tumor destruction provides a much more direct way of *in situ* DC targeting, without the need for retroviral infection or construction of recombinant proteins.

The combination of ablation and CpG-ODN showed promising results in the mouse system used. However, we note that, as TLR expression on DCs is different between species, analysis of different TLR ligands in combination with ablation will be important to allow translation to cancer patients. Possibly, TLR3 or TLR7/8 triggering may be more potent in the activation of human DCs (53). Furthermore, it might be rewarding to consider the effects of different TLR stimuli on other parts of the immune system. For instance, it was recently shown that the TLR2 ligand Pam_3cys was also able to abrogate regulatory T cell–mediated suppression of T cell expansion (54). TLR stimulation could also affect natural killer cells and other leukocytes. Activated natural killer cells have been shown to promote DCs that have captured antigen, and vice versa, DCs have been shown to promote the proliferation of natural killer cells and increase their cytotoxic activity (55, 56). Intratumoral injection of different TLR ligands following *in situ* tumor destruction may thus be aimed at enhancing DC activation, but also at activating other cells. Targeting DCs as well as other parts of the immune system may result in an increasingly effective antitumor response.

Collectively, these data show that *in vivo* tumor destruction, in combination with CpG-ODN administration, creates a unique and

potent *in situ* DC vaccine. The fact that both treatment modalities are currently applied separately in cancer patients makes this promising "*in vivo* DC vaccine" readily applicable in clinical settings.

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Synergy between *In situ* Cryoablation and TLR9 Stimulation Results in a Highly Effective *In vivo* Dendritic Cell Vaccine

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