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ANTI-TUMOR ACTIVITY MEDIATED BY CpG: THE ROUTE OF ADMINISTRATION IS CRITICAL

Yanyan Lou¹, Chengwen Liu¹, Gregory Lizée¹, Weiyi Peng¹, Chunyu Xu¹, Yang Ye¹, Brian A. Rabinovich², Yared Hailemichael¹, Alexander Gelbard¹, Dapeng Zhou¹, Willem W. Overwijk¹, and Patrick Hwu¹

¹Departments of Melanoma Medical Oncology, The Center for Cancer Immunology Research, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

²Departments of Experimental Diagnostic Imaging, The Center for Cancer Immunology Research, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Abstract

Unmethylated CpG oligodeoxynucleotides (CpG) are synthetic Toll-like receptor 9 agonists that activate innate immune cells and which have been tested as an immune therapy in a number of cancer clinical trials. Although some anti-tumor immune responses have been reported, so far the majority of studies have failed to demonstrate significant clinical responses to CpG. Here we demonstrate that the route of administration is critical to the anti-tumor activity of CpG. Although intravenous (i.v.) injection of CpG was capable of inducing the activation and expansion of tumor antigen-specific T cells, most of these activated T cells failed to migrate to tumor sites. By contrast, intratumoral (i.t.) injection of CpG led to extensive tumor infiltration of antigen-specific T cells and subsequent tumor suppression. We further demonstrate that very high levels of inflammatory chemokines (RANTES, IP10, JE, MCP5, MIP1 α and MIP1 β) were induced in the tumor microenvironment following i.t. CpG injection, compared to administration via the i.v. route. Interestingly, *in vivo* depletion of plasmacytoid dendritic cells (pDC) greatly reduced the levels of chemokines induced; also, T cell accumulation and anti-tumor effect were impaired. We also demonstrate that i.t. but not i.v. CpG injection induced a broad antigen-specific T cell response against tumor-derived antigens. Collectively, our data provides evidence that the route of CpG administration is a critical factor in mediating antitumor activity. By inducing localized inflammatory signals at tumor sites, i.t. CpG effectively promotes the migration, activation and function of immune cells, ultimately leading to improved tumor control.

Requests for reprints: Dr. Patrick Hwu, Department of Melanoma Medical Oncology, Unit 430, The Center for Cancer Immunology Research, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-563-1728; Fax: 713-745-1046; phwu@mdanderson.org.

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Keywords

CpG-ODN; plasmacytoid dendritic cell (pDC); Adoptive cell transfer (ACT)

INTRODUCTION

The immune system is largely successful at controlling or eliminating viral infections, but is comparatively ineffective at preventing the outgrowth of tumors or eliminating metastatic deposits. A critical difference between anti-viral immune responses and anti-tumor responses is the recognition of pathogen-associated molecular patterns (PAMPs) by innate immune cells through toll-like receptors (TLR). TLR agonists possess the ability to generate strong innate and adaptive immune responses in various disease settings, including cancer. Among these, TLR9 agonists such as unmethylated CpG oligodeoxynucleotides (CpG-ODN, hereafter referred to as CpG) have been shown to specifically activate plasmacytoid dendritic cells (pDC) and B cells in humans, in addition to myeloid conventional dendritic cells (cDC) and macrophages in mice. Through binding to TLR9, CpG has been shown to promote DC maturation, antigen presentation and production of pro-inflammatory cytokines, leading to enhanced T cell-mediated anti-tumor immune responses (1, 2).

A variety of studies evaluating CpG as a cancer vaccine adjuvant have been performed in animal models and in clinical trials. In this context, CpG has been demonstrated to activate natural killer (NK) cell cytotoxicity and strongly increase antigen-specific T-cell responses to peptide or protein cancer vaccines (3, 4). However, most of these studies used a vaccine formulation that combined CpG with tumor antigens, and were administered via a subcutaneous (s.c.) route. Although T-cell responses were enhanced in peripheral blood following CpG treatment, in most patients this treatment failed to induce significant tumor regressions. Recently, a randomized phase III clinical trial using intravenous (i.v.) injection of CpG in combination with chemotherapy vs. chemotherapy alone for non-small cell lung cancer failed to demonstrate any beneficial effects from CpG (2).

We have shown that intratumoral (i.t.) injection of CpG-activated pDC can induce tumor regression via NK cell activation and tumor antigen-specific T cell responses (5). Here we report that the route of administration is a critical factor for determining the anti-tumor activity of CpG. Using a murine melanoma tumor model we found that, unlike other modes of administration, i.t. injection of CpG induced strong inflammation within the tumor microenvironment that stimulated not only the afferent limb, but also the efferent limb of the adaptive anti-tumor immune response. Intratumoral CpG resulted in the infiltration of tumor antigen-specific T cells, and NK cells, and we provide evidence that pDCs play an important role in the initiation of this process. Therefore, we conclude that CpG administered i.t. can act as a potent inflammatory mediator for attracting and activating innate and adaptive immune cells to induce therapeutic anti-tumor activity.

MATERIALS AND METHODS

Cell lines, Reagents and Mice.

B16 melanoma cells and human gp100 transduced MC38 murine colon adenocarcinoma cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, L-glutamine, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin (all from Invitrogen/Life Technologies, Inc., Rockville, MD). CpG-ODN-2216 was synthesized by Invitrogen Life Technologies. Anti-mouse monoclonal antibodies used for flow cytometry analysis were purchased from BD Biosciences (San Jose, CA). Female C57BL/6 mice were purchased from the NCI (Bethesda, MD). C57BL/6J-Tyr-2J/J albino mice were purchased from the Jackson Laboratory. Pmel-1 transgenic mice express a TCR specific for an H-2Db-restricted epitope of the melanoma tumor antigen gp100 (gp100₂₅₋₃₃) on a C57BL/6 background as described previously (6, 7). All mice were maintained in a pathogen-free animal facility at the M.D. Anderson Cancer Center. Mice were used at 8–10 weeks of age, and all animal work was performed according to NIH and institutional guidelines.

Tumor treatment and monitoring.

C57BL/6 mice (5 mice per group) were subcutaneously inoculated with $3 - 5 \times 10^5$ B16 tumor cells on day -7. Tumor-bearing mice were treated on day 0 by i.v. injection of $3 - 7 \times 10^6$ splenocytes derived from pmel-1 transgenic mice or 3×10^6 cultured pmel-1 T cells as described previously (4). Mice also received s.c. vaccination with gp100/IFA. 30–50 µg of CpG was injected either i.t. or i.v. Tumor growth was monitored by measuring the perpendicular diameters of tumors.

Transduction of Mouse T Cells and In Vivo Bioluminescence Imaging.

Splenocytes from pmel-1 mice were cultured and transduced as described before (8). For *in vivo* imaging studies, in order to visualize luciferase-expressing T-cells within a tumor, a non-pigmented tumor is required. Therefore, we utilized the MC38-gp100 tumor model. Isoflurane-anaesthetized animals were imaged using an IVIS 200 system (Xenogen) 8 min after i.p. injection of 2 mg of D-luciferin, according to the manufacturer's specifications. Living Image software was used to analyze the data. Regions of interest (ROI) were manually selected over relevant regions of signal intensity. The quantification is reported as the average of photon flux within ROI.

Tumor Antigen-Specific T-cell Response.

B16 tumor-bearing mice were sacrificed 6 days after injection of CpG. Splenocytes were harvested, and erythrocytes were depleted using ACK lysing buffer. Cells were stimulated with or without 10 µM P15E in the presence of Brefeldin A for 6 hrs. Cells were collected and incubated with rat anti-CD16/32 mAb (2.4G2) to block non-specific binding, and then stained with anti-CD3e, anti-CD8α and intracellular IFN-γ according to the manufacture's instruction (BD Bioscience). The percentage of antigen-specific T cells in tumor was analyzed using flow cytometry.

pDC depletion.

The 927 hybridoma cell line was kindly provided by Dr Marco Colonna, Washington University School of Medicine (9). The monoclonal antibody 927 was purified by protein G affinity chromatography. For pDC depletion, tumor-bearing mice were injected intraperitoneally with 500µg of antibody 927 before and during treatment. Rat IgG was used as control.

Chemokine protein arrays.

B16 tumor-bearing mice were sacrificed 24 hours after injection of CpG, and tumors were resected and weighed. Tumors were homogenized and sonicated in lysis buffer containing protease inhibitors. Cleared tumor lysates after centrifugation were tested using searchlight chemokine protein array according to the manufacturer's protocol (Aushon Biosystems, Woburn, MA).

Statistical Analysis.

The statistical analyses to compare tumor size, the percentage and absolute number of cells and chemokine levels were determined using the Mann-Whitney nonparametric U test or a Student T test. *P*-values are based on 2-tailed tests, with *P* < 0.05 considered statistically significant. The Kaplan-Meier test was used to compare mouse survival between groups.

RESULTS

Intratumoral CpG synergized with adoptive T-cell transfer to induce strong anti-tumor activity.

CpG, through binding to TLR9 expressed on DCs and B cells, plays a critical role in triggering both innate and adaptive immune responses. To determine whether CpG was capable of enhancing cancer vaccine-mediated anti-tumor activity *in vivo*, we studied murine B16 melanoma, an aggressive tumor with low immunogenicity, as a tumor model. Mice were inoculated subcutaneously with B16 tumor cells, and established tumors were treated 7 days later with adoptive transfer of pmel-1 T cells, transgenic T cells that express a T-cell receptor (TCR) specifically recognizing an H-2Db–restricted epitope of gp100, in conjunction with gp100 peptide/IFA vaccination (7). Intratumoral (i.t.) injection of CpG in PBS or PBS control was given twice per week and tumor growth was monitored. As shown in Fig. 1, treatment of mice with either CpG alone or adoptive T-cell transfer (ACT) with peptide vaccination alone failed in delaying tumor growth. However, i.t. injection of CpG combined with ACT/gp100 vaccination led to a significant inhibition of tumor growth (Fig. 1A), with concomitant enhanced survival (Fig. 1B). In light of the more limited clinical application of intratumoral administration, we examined whether intravenous (i.v.) injection of CpG could also induce effective anti-tumor activity in a total body irradiation (TBI) treatment setting. However, while i.t. administration of CpG in the context of ACT significantly delayed B16 tumor growth and enhanced mice survival, i.v. administration of CpG did not, demonstrating that the route of CpG administration was a critical factor in determining treatment efficacy (Fig. 1C, Fig. 1D).

Intratumoral CpG treatment led to enhanced T-cell infiltration of tumors.

To understand the differences in anti-tumor activity observed between the i.t. and i.v. routes of CpG administration, mice were adoptively transferred with pmel-1 T cells expressing firefly luciferase, followed by gp100 peptide vaccination and CpG treatment as described previously (8). Following i.t. or i.v. CpG treatments, the distribution and migration patterns of pmel-1T cells *in vivo* over time were assessed by bioluminescence imaging (BLI) on daily basis. Although transferred pmel-1 T cells were detectable at tumor sites in all groups, mice receiving i.t. CpG treatment displayed much stronger luciferase signal than mice receiving i.v. CpG treatment or control PBS (Fig 2A). The average luciferase intensity in tumors from each group was quantified using live imaging detection software. As shown in Fig. 2B, i.t. administration of CpG led to 5–10 fold higher luciferase intensity, which is proportional to the number of tumor antigen-specific pmel-1 T cells infiltrating tumors. In clear contrast, treatment with i.v. CpG did not change the migration of antigen-specific T cells to tumor. Meanwhile the percentage of pmel-1 T cells was not significantly different in peripheral blood among the groups (Fig. 2C). The enhanced tumor infiltration of antigen-specific T cells induced by i.t. CpG treatment was observed for up to 10 days, indicating that the inflammatory effect could be sustained in the tumor microenvironment following i.t. CpG injection.

We also used flow cytometry to analyze tumor infiltration of both adoptively transferred pmel-1 CD8⁺ T cells and endogenous bystander CD8⁺ T cells. In this setting, we found that treatment of mice with i.t., but not i.v., CpG in combination with ACT/peptide vaccination induced a 5-fold increase of infiltration of adoptively transferred pmel-1 CD8⁺ T cells (Fig. 3A). Interestingly, we also observed similar level of increase in migration of endogenous bystander T cells (Fig. 3B). Thus, these data demonstrate that, compared with i.v. administration, i.t. CpG treatment can induce enhanced tumor infiltration by diverse CD8⁺ T-cell populations, including both adoptively transferred and endogenous CD8⁺ T cells. This observation likely explains why enhanced anti-tumor activity was observed only in mice receiving i.t. CpG treatment.

Intratumoral administration of CpG induced dramatic increases in proinflammatory chemokines.

The rapid influx of CD8⁺ T cells into tumor tissues following injection of CpG suggested that this treatment may induce the production of lymphocyte-attracting chemokines within the tumor microenvironment. To explore this hypothesis, large, established B16 tumors were harvested 24 hours following i.t. or i.v. CpG injection and the levels of chemokines present were analyzed. As shown in Fig. 4, injection of CpG through intratumoral administration dramatically increased the levels of chemokines including IP10, RANTES, JE, MCP5, MIP1 α and MIP1 β within the tumor microenvironment, 5- to 1000-fold higher as compared to i.t. PBS. Of the induced chemokines, the level of RANTES was the most strikingly increased, to nearly 3 logs above that of controls. Most of these induced chemokines are proinflammatory, and exert chemotactic activities on T cells, natural killer (NK) cells, and dendritic cells (10–12). In particular, RANTES has been shown in a number of other studies to induce to enhance tumor infiltration of CD8⁺ T cells and delay tumor growth (13–15).

To determine whether enhanced T cell accumulation at the tumor site was also due to the increased expression of adhesion molecules such as E-selectin and P-selectin, we also performed immunohistochemistry of tumors to stain for these molecules. These experiments, however, could not detect significant changes in the expression of P-selectin and E-selectin within the tumor microenvironment following i.t. CpG treatment, indicating that augmented T-cell accumulation in the tumors was probably not mediated by increased expression of these adhesion molecules (data not shown).

Plasmacytoid dendritic cells mediated the chemokine production induced by intratumoral CpG treatment.

To better understand the mechanism by which high levels of inflammatory chemokines were induced following i.t. CpG treatment, we next examined cell types known to play a role in CpG mediated chemokine production. In previous studies, we have shown that pDC produce very high amounts of chemokines such as RANTES, IP10, MIP1 α and MIP1 β upon CpG stimulation(5); by contrast, stimulated cDC expressed RANTES, MIG, I-TAC, MIP-3 β , and BCA-1 (16). To determine whether pDCs were responsible for CpG-mediated chemokine production in our tumor model, pDCs were depleted before and during treatment. As shown in Fig. 5A, the levels of chemokines IP10, RANTES, JE, MCP5, MIP1 α and MIP1 β within the tumor microenvironment were dramatically reduced in mice depleted of pDC, indicating that these cells were required for the chemokine induction mediated by i.t. CpG treatment. To further understand if T cell accumulation and anti-tumor effects were dependent on pDC, antibody-based depletion of pDCs was performed. As shown in Fig. 5B and Fig. 5C, T cell accumulation and anti-tumor effect were partially impaired by the pDC depletion.

To explore the possibility that increasing the dose of i.v.-administered CpG could increase the local tumor concentration and achieve effects comparable to i.t. CpG, we compared the level of chemokines at the tumor site following i.v. administration of low (50 μ g) and high (500 μ g) concentrations of CpG. As shown in Fig. 5A, increasing the i.v. dose 10-fold failed to increase the levels of chemokines in the tumor. This demonstrated that simply increasing the amount of i.v. administrated CpG could not recapitulate the effect of using a much lower dose administered by the i.t. route.

Intratumoral injection of CpG induced broad CTL response toward tumor antigen released by tumor lysis.

Next, we asked whether i.t. CpG could induce the *de novo* priming of CD8⁺ T-cells against tumor antigens not directly recognized by transferred pmel-1 CD8 cells. We chose to study a natural tumor antigen expressed by B16 cells, p15E (17), an endogenous retroviral envelope protein expressed by multiple murine tumors. Six days after the i.t. or i.v. injection of CpG, spleens were harvested from B16 tumor-bearing mice. Splenocytes were stimulated in the presence or absence of p15E peptide for 6 hrs, and p15E antigen-specific CTL were examined by detection of IFN- γ secreting CD8⁺ T cells using intracellular staining. As shown in Fig. 6A, only i.t. CpG led to the induction of p15E specific CTL, whereas i.v. CpG administration led to no detectable *de novo* T-cell priming. As above, even administering significantly higher doses of CpG via the i.v. route failed to induce T-cell priming against endogenous tumor antigens. To further understand the mechanism behind this, spleens,

peripheral lymph nodes (PLN), tumor draining lymph nodes (DLN) and tumors were harvested from B16 tumor-bearing mice 24 hrs after CpG injection. CD40 and CD86 expression was assessed as a measure of DC maturation. As shown in Fig. 6B, i.t. CpG facilitated the activation of DCs in DLN, but i.v. CpG facilitated the activation of DCs in spleen and PLN. This observation may explain why endogenous antigen specific T-cell responses could only be induced by i.t. CpG treatment.

DISCUSSION

As a class of compounds with potent immunostimulatory effects, TLR agonists have been extensively studied in animal and pre-clinical settings for the treatment of multiple diseases (18–20). CpG, a well documented TLR9 agonist that induces a potent innate and adaptive immune response, is currently being investigated in clinical trials for use as a vaccine adjuvant in the treatment of infections, asthma, allergy and cancer (19, 21, 22). TLR9 is mainly expressed by pDC and B cells, and localizes to the endosomal and lysosomal compartments of these cells (23, 24). Upon stimulation through TLR9, CpG can drive pDC to become activated and mature, and to produce very high amounts of type I IFN, which in turn activates multiple other immune cell types including monocytes, NK cells, myeloid dendritic cells, macrophage and T cells (25). Stimulation of TLR9 on B cells induces activation, differentiation, proliferation, and enhanced antibody production (26). Given the potent immunostimulatory effects of CpG, tumor immunologists have shown great interest in using CpG as a monotherapy agent, vaccine adjuvant or in combination with chemotherapy, antibodies or cytokines to boost immune responses against cancer.

Although pre-clinical CpG studies have demonstrated success in enhancing anti-tumor T-cell responses, most treatment regimens using CpG as a monotherapy failed to induce regression of established tumors. In combination with other treatment therapies such as chemotherapy, radiation therapy or monoclonal antibodies, however, CpG enhanced response rates, indicating a potential synergistic effect of CpG as a cancer treatment adjuvant (27, 28). In experimental cancer studies, several routes of CpG administration have been tested, including intravenous, intratumoral, peritumoral and subcutaneous. Although some administration routes appear better than others at inducing T-cell responses, a side-by-side comparison of different routes in this setting has not, to our knowledge, been reported.

Given the potential importance of administration route in designing optimal cancer treatment strategies, in this study we carefully evaluated the anti-tumor efficacy of i.v. CpG treatment compared with i.t. treatment in the context of an ACT-based treatment regimen. We demonstrated that the route of CpG administration is a critical factor for the success of treatment, and that i.t. CpG is clearly superior to i.v. CpG treatment. Our data provide evidence that i.t. CpG administration creates a strong inflammatory environment at tumor sites by activating tumor-infiltrating pDC. Upon activation, pDC rapidly produce large amounts of type I IFNs, which in turn activate NK cells, B cells, T cells, and cDCs, leading to the induction of innate and adaptive immune responses (25, 29–32). In addition to these functions of activated pDC, our data have also shown another important contribution by pDCs in the antitumor response, the ability to produce pro-inflammatory chemokines at tumor sites. Large amounts of chemokines RANTES, JE, MCP5, MIP1 α , MIP1 β and IP10

were produced within the tumor microenvironment following i.t. CpG treatment, and pDC depletion *in vivo* strongly abrogated this induction. In addition to partially impairing T-cell accumulation and anti-tumor effects.

Although pDC can be activated by CpG using most administration routes, s.c. administration of CpG has been the preferred method in many studies owing to its slower absorption and high levels of drug accumulation in the draining lymph nodes (33–35). Our data demonstrate that i.t. CpG, which in melanoma tumors is analogous to s.c. administration, showed significant advantages over i.v. administration by creating inflammatory signals directly within the tumor microenvironment. Recognition of PAMPs at tumor sites alerts the innate immune system to subsequently attract multiple immune cells that function in the anti-tumor response. Recent studies have demonstrated that tumors highly infiltrated with lymphocytes also express higher levels of JE, MIP-1 α , MIP-1 β and, in particular, RANTES, which correlated positively with the level of T cells infiltrating individual tumors (36, 37). These findings are in accordance with our observation that i.t., but not i.v., CpG could induce extensive tumor infiltration by antigen-specific T cells, resulting in strong anti-tumor activity.

Selective delivery of CpG and other immuno-drugs to tumor sites remains an important goal of cancer immunotherapy. There have been reports of conjugating CpG to tumor-binding antibodies (38). However, the uptake of antibodies by mononuclear phagocyte system unavoidably causes the release of CpG at non-tumor sites. Poor intratumoral distribution of some tumor-binding antibodies is another barrier. Tumor specific ligands, such as peptide analogs to melanocyte stimulating hormone, have shown some promise in selective accumulation in tumor sites when administered intravenously (39). Nanoparticles and nanopolymers which selectively accumulate in tumor sites through enhanced permeability and retention (EPR) effect and a recently discovered M2 macrophage uptake mechanism (40), are another class of interesting cargos for CpG and other immunotherapeutics.

In conclusion, our data suggest that systemic administration of CpG may not be the most efficient approach to induce anti-tumor immunity and tumor regression. Intratumoral administration can lead to tumor shrinkage and induce increased levels of tumor-specific T cells which can subsequently exert systemic anti-tumor control. Approaches that target CpG and other proinflammatory molecules specifically to tumors are a rational approach to increasing anti-tumor activity, both as monotherapy and in combination with anti-tumor vaccination and/or adoptive T cell therapy.

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Figure 1. Intratumoral CpG synergizes with adoptive T-cell transfer to induce strong anti-tumor activity.

(A), (B) Synergistic effect of the combination of ACT with i.t. CpG. C57BL/6 mice (5 mice per group) were subcutaneously inoculated with B16 tumor cells on day -7 and treated on day 0 by i.v. injection of pmel-1 T cells and s.c. vaccination with gp100/IFA. CpG was injected on days 0, 4 and 7. (C), (D) i.t. but not i.v. CpG injection enhances the anti-tumor effect of ACT. B16 tumor-bearing mice were irradiated with 350 cGy TBI on day -1 and on day 0 were treated with cultured pmel-1 T cells i.v. and s.c. vaccination with gp100/IFA followed by i.t. or i.v. CpG on days 0, 4 and 7. Recombinant human IL-2 was administered i.p. for 3 days after T cell transfer (600,000 I.U. twice daily). Tumor growth is shown as

mean $\pm$ SEM of the perpendicular diameters of tumors and are representative of three independent experiments with similar results. (** $P < 0.01$ for i.t. CpG versus i.v. CpG or i.t. PBS).

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Figure 2. I.t. CpG induces accumulation of adoptively transferred pmel CD8 T cells at the tumor site.

C57BL/6 albino mice were implanted s.c. with 5×10^5 MC38/gp100 tumor cells on day -7, irradiated with 350 cGy TBI on day -1 and treated on day 0 by i.v. injection of 3×10^6 in vitro expanded OFL (luciferase)-expressing pmel-1 T cells and s.c. vaccination with gp100/IFA. 50 μ g CpG was injected either i.t. or i.v. on day 0, 4 and 7. Recombinant human IL-2 was administered as described in Fig. 1. (A) *In vivo* trafficking of OFL-expressing pmel-1 T cells. Isoflurane-anaesthetized animals were imaged 8 min after i.p. injection of 2 mg of d-luciferin on day 5. The quantification is reported as the average of photon flux within ROI.

(B) Summary of quantitative imaging analysis of transferred T cells in tumor site. (* $P < 0.05$, ** $P < 0.01$ for i.t. CpG versus i.v. CpG or i.t. PBS). (C) Percentage of transferred pmel-1 T cells in blood. (ns $P > 0.05$ for i.t. CpG versus i.v. CpG or i.t. PBS). Data are representative of three independent experiments.

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Figure 3. I.t. CpG induces accumulation of both adoptively transferred T cells and non-transferred T cells at the tumor site.

C57BL/6 mice (5 mice per group) were inoculated s.c. with B16 tumor cells on day -7 and treated on day 0 by i.v. injection of in vitro expanded pmel-1 T cells and s.c. vaccination with gp100/IFA. CpG was injected on days 0, 4 and 6. Mice were sacrificed on day 7. Percentage of tumor-infiltrating transferred T cells (A) and non-transferred T cells (B) were calculated by gating on total living lymphocytes. (* $P < 0.05$ for i.t. CpG versus i.v. CpG or i.t. PBS). Data are representative of three independent experiments.

Figure 4. I.t. CpG induces proinflammatory chemokines.

B16 tumor-bearing mice were sacrificed 24 hours after injection of CpG. Tumors were homogenized and sonicated in lysis buffer containing protease inhibitors. Cleared tumor lysates after centrifugation were tested using Searchlight chemokine protein array according to the manufacturer's protocol. (* $P < 0.05$, ** $P < 0.01$ for i.t. CpG versus i.v. CpG or i.t. PBS). Data are representative of two independent experiments.

Figure 5. Plasmacytoid dendritic cells mediate the chemokine production induced by i.t. CpG treatment.

(A) B16 tumor-bearing mice were pDC-depleted by 500 μ g antibody 927 i.p. on day -2, -1, and 6 hrs before CpG injection and sacrificed 24 hours after CpG injection. Samples were tested as described in Fig. 4. (* $P < 0.05$, ** $P < 0.01$ for i.t. CpG versus i.v. CpG or i.t. PBS).

(B) Mice bearing 7-day B16 tumors were treated as in Fig. 2; pDC were depleted by i.p. injection of 500 μ g antibody 927 on day -2, -1, 0, and 3. CpG was injected on day 0 and 4. Mice were sacrificed and tumors were harvested on day 5. Quantitation of pmel-1 T cell tumor infiltration was assessed by flow cytometry analysis. (C) Mice bearing 7-day B16

tumors were treated as in Fig 2; pDC were depleted by intraperitoneally administered 500 μ g antibody 927 on day -2, -1, 0, 3 and 6. CpG was injected on day 0, 4 and 7. Tumor growth was monitored by measuring the perpendicular diameters of tumors. Data are representative of two independent experiments.

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Figure 6. I.t. CpG induces broad CTL response toward tumor antigen released by tumor lysis. (A) Spleens were harvested from B16 tumor-bearing mice 6 days after the i.t. or i.v injection of CpG. Splenocytes were stimulated in the presence or absence of p15E peptide for 6 hrs and antigen specific CTL were examined by detection of IFN- γ -secreting CD8 T cells using intracellular staining. (B) Spleens, PLN, DLN and tumors were harvested 24hrs after injection of CpG. After isolation, leukocytes were stained with CD11c, CD40 and CD86 for

flow cytometry analysis. Data are representative of two independent experiments. (* $P < 0.05$ for i.t. CpG versus i.v. CpG or i.t. PBS).

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