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Anti-tumor activity of CpG-ODN aerosol in mouse lung metastases

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Studies in preclinical models have demonstrated the superior anti-tumor effect of CpG oligodeoxynucleotides (CpG-ODN) when administered at the tumor site rather than systemically. We evaluated the effect of aerosolized CpG-ODN on lung metastases in mice injected with immunogenic N202.1A mammary carcinoma cells or weakly immunogenic B16 melanoma cells. Upon reaching the bronchoalveolar space, aerosolized CpG-ODN activated a local immune response, as indicated by production of IL-12p40, IFN-γ and IL-1β and by recruitment and maturation of DC cells in bronchoalveolar lavage fluid of mice. Treatment with aerosolized CpG-ODN induced an expansion of CD4+ cells in lung and was more efficacious than systemic i.p. administration against experimental lung metastases of immunogenic N202.1A mammary carcinoma cells, whereas only i.p. delivery of CpG-ODN provided anti-tumor activity, which correlated with NK cell expansion in the lung, against lung metastases of the poorly immunogenic B16 melanoma. The inefficacy of aerosol therapy to induce NK expansion was related to the presence of immunosuppressive macrophages in B16 tumor-bearing lungs, as mice depleted of these cells by clodronate treatment responded to aerosol CpG-ODN through expansion of the NK cell population and significantly reduced numbers of lung metastases. Our results indicate that tumor immunogenicity and the tumor-induced immunosuppressive environment are critical factors to the success of CpG therapy in the lung, and point to the value of routine sampling of the lung immune environment in defining an optimal immunotherapeutic strategy.

Tumor Immunology

Synthetic oligodeoxynucleotides containing dinucleotides with unmethylated CpG motifs (CpG-ODN), agonists of TLR9, represent a novel approach to stimulating an effective anti-tumor response as demonstrated in preclinical models[1] and in patients with malignant melanoma, renal carcinoma and recurrent or refractory lymphoma.[1,2] However, efficacy of CpG in the lung, where virtually continuous exposure to environmental antigens dictates local immunological homeostasis and the meticulous control of immune responses, has been limited to the control of lung metastatic growth only when administered before tumor cell injection or immediately after when disease is minimal in various preclinical tumor models.[3–5] Although a randomized Phase II trial with non-small lung cancer patients showed promising results and led to two large randomized Phase III trials comparing CpG-ODN plus chemotherapy to chemotherapy alone, interim analysis of those trials did not support the promise of CpG-ODN in enhancing the efficacy of chemotherapy and caused the suspension of the trials.[2] Several studies have suggested that the route of administration is a critical factor in influencing CpG activity. The anti-tumor effect of CpG has been optimized by direct injection into the tumor to enable increased local concentrations in the tumor microenvironment and to ensure effective local activation of both innate and adaptive immune responses.[6–9]

Thus, local instead of systemic administration of the TLR9 agonist might represent an option to improve the efficacy of CpG as adjuvant in protecting against lung cancer or in treating and/or preventing lung metastases derived from different primary tumors. Bronchial and bronchoalveolar

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What's new?

CpG oligodeoxynucleotides (CpG-ODN) have anti-tumor activity when given at the tumor site. In this study, the authors asked whether aerosol delivery of CpG-ODN might be more effective than systemic delivery against lung metastases in mice. It was indeed more effective against metastases of immunogenic carcinoma cells. However, this was not the case against metastases of poorly-immunogenic B16-melanoma cells. The results indicate that aerosol delivery may be a valuable, practical approach to CpG therapy in the lung, but that tumor immunogenicity and tumor-induced immunosuppression are critical factors in its success.

tumors are accessible *via* the endobronchial space. To date, CpG-ODN has been delivered to the lung intranasally (i.n.) in different preclinical studies that document its adjuvant activity and therapeutic potential in the treatment of airway pathogen infections and in asthma models.[10–12] The immunomodulatory effects triggered locally in the lung by CpG-ODN through this route have recently been well studied to clarify the mechanisms involved in the CpG-linked adjuvanticity.[13] Although this route is not practical in the clinical setting, an inhaler/nebulizer represents a more convenient and simpler approach for repeated application, ensuring a more uniform distribution and penetration into the alveolar region of the lung. Aerosol administration to deliver therapeutic agents locoregionally, including gene delivery of mitogen-activated protein kinase Kinase 4,[14] PTEN,[15] interleukin-12,[16] interleukin-2,[17] granulocyte/macrophage colony-stimulating factor[18] and p53,[19] and delivery of chemotherapeutic agents, such as gemcitabine[20] and docetaxel combined with celecoxib,[21] and antisense oligonucleotides alone or in combination with anticancer drugs,[22,23] have also been tested in mice with lung cancer, and a Phase I clinical trial with aerosolized liposomal 9-nitro-20 (S)-camptothecin in lung cancer patients has been performed.[24] Overall, these studies indicate that aerosolized pulmonary delivery allows wide localization of these agents in the bronchiolar epithelium, alveolar epithelium and endothelium and, as compared to systemic administration, provides better retention of the different agents in the lungs while minimizing their penetration into the systemic circulation, thus limiting adverse side effects.

Herein, we evaluated the ability of aerosol-delivered CpG-ODN to activate immune cells in the lung and its efficacy in treating established malignant lung lesions in two different murine tumors, the immunogenic mammary carcinoma N202.1A expressing the rat neu oncogene, and the poorly immunogenic B16 melanoma in which protection is mainly mediated by innate effectors.

Material and Methods

Cell lines and reagents

N202.1A cells, derived from a spontaneous mammary carcinoma in an FVB-neuN transgenic mouse,[25] and B16 mouse melanoma cells were routinely maintained at 37°C in a 5% CO_2 atmosphere in Roswell Park Memorial Institute medium 1640 and Dulbecco's modified Eagle's Medium, respectively, supplemented with 10% fetal calf serum and 2 mM glutamine. Purified, phosphorothioated ODN1826 (5′-TCCATGACGTT CCTGACGTT-3′) containing CpG motifs was synthesized by TriLink Biotechnologies (San Diego, CA).

Mice and experimental protocols

All experiments were carried out using 8- to 12-week-old female FVB and C57BL/6 mice (Charles River, Calco, Italy) maintained in laminar-flow rooms at constant temperature and humidity, with food and water given *ad libitum*. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan according to institutional guidelines.

Aerosol administration was performed using a mouse whole-body exposure system consisting of an Aeroneb® Lab Micropump Nebulizer that generates aerosol into a plastic box with a sealed top and a wire netting floor that contains up to 10 mice (EMMS Unit 32 Bordon, Hants, UK). CpG-ODN was dissolved in 5 ml saline and placed in the nebulizer unit. The aerosolized solution was introduced into the box *via* a accordion tube at a short distance from the nebulizer at one end and discharged at the other end. Mice were exposed to aerosol for 15 min, with the 5-ml volume of liquid in the nebulizer nearly consumed in 10 min. Control mice received aerosolized saline. Intraperitoneal (i.p.) administration was performed as described[6] using 20 μg CpG ODN in 200 μl saline/mouse. FVB or C57BL/6 mice were injected intravenously (i.v.) with 3×10^5 N202.1A carcinoma cells or 5×10^5 B16 melanoma cells, respectively, and treated 72 hr later with aerosol (1.5 or 2.5 mg) or i.p. CpG-ODN administered at 72- to 96-hr intervals for 3 weeks. Control mice were injected with tumor cells only. All mice were anesthetized and euthanized 5 weeks after tumor injection, and macroscopic lung metastases were counted. In each experiment, mice were weighed twice weekly.

Depletion of macrophages with clodronate

Clodronate-loaded liposomes or control PBS-liposomes were prepared as described[26] and injected (100 μl) intratracheally (i.t.) into anesthetized mice 96 hr after i.v. injection of 5×10^5 B16 melanoma cells. After 24 hr (120 hr after tumor injection), mice were treated with aerosol or i.p. CpG-ODN 4 days/week for 2 or 4 weeks in experiments to evaluate lung infiltrate or protection from tumor growth, respectively. In

each experiment, flow cytometry using cellular infiltrates from digested lungs of mice treated with Cl_2MDP-liposomes revealed that 70%–80% of alveolar macrophages were depleted 72 hr after Cl_2MDP injection, as compared to PBS-liposome-treated mice (3 mice/group).

Statistical analysis

Analyses were performed using GraphPad Prism 5 (GraphPad Software). For studies comparing differences between two groups, the unpaired Student's *t* test was used. For differences between more than two groups, statistical significance was determined using one-way Anova test, followed by a Dunnet's or Tukey's post-test for comparison between groups.

Results

Effects of CpG-ODN aerosolization in BAL fluid

The efficacy of aerosolized CpG-ODN in reaching the lung and inducing an immune response was evaluated in BAL fluid 24, 48 and 72 hr after aerosol treatment with 5 ml of saline containing 0.5, 1.5 or 2.5 mg of oligonucleotide 1826 (5-10 FVB mice in the same aerosol box). Production of IL-12p40, which has been shown to be the most increased cytokine after i.n. CpG-ODN treatment,[13] was threefold higher in mice treated with 1.5 or 2.5 mg of CpG-ODN *versus* saline-treated control mice and remained high even after 48 and 72 hr, while no significant increase in IL-12p40 was detected with the dose of 0.5 mg (Fig. 1*a*). No modulation of IL12p40 concentration was observed in sera of mice treated at the different doses of CpG-ODN as compared to that in control mice (data not shown). A similar production of IL-12p40 was observed in experiments using C57BL/6 mice (data not shown).

Proteomic analysis of a panel of pro-inflammatory cytokines at 24, 48 and 72 hr after aerosol administration at the selected dose of 1.5 mg CpG-ODN also revealed a significant increase in IL-1β and IFN-γ (Fig. 1*b*) but no significant modulation of IL-2, IL-4, IL-5, IL12p70, TNF-α or IL-6 in the BAL fluids.

Analysis of DC cells (CD45+CD11c+ F4/80-), which are selectively recruited by i.n.-delivered CpG in the bronchoalveolar space,[13] revealed no significant changes in the percentage of these cells at 24, 48 and 72 hr in BAL fluid recovered from mice treated once with aerosolized CpG-ODN at 0.5, 1.5 or 2.5 mg/5 ml saline (data not shown); however, repeated treatments with 1.5 mg CpG-ODN at intervals of 72–96 hr for 2 weeks induced a significant increase in the percentage of this population in BAL fluid as compared to control mice (Fig. 1*c*) and enhanced maturation of these cells, as indicated by the significant increase in expression levels of the activation marker CD86 (Fig. 1*d*). This treatment schedule also induced high production of IL-12p40 in BAL fluid (Fig. 1*e*).

Unlike i.n.-administered CpG-ODN, which induces lung tissue inflammation associated with weight loss in rodents,[27–29] prolonged repeated treatments at intervals of 72-96 hr with 1.5 mg CpG-ODN aerosol for 3 weeks were well tolerated. No effects on body weight and no histological changes in the structure of lungs, as indicated by histopathological examination of hematoxylin and eosin-stained sections of lung tissue, were observed in mice exposed to CpG-ODN aerosolization (data not shown). Together, the results indicate that aerosolized CpG-ODN can reach the bronchoalveolar space in the lung and locally activate an innate immune response without apparent signs of toxicity.

Anti-tumor activity of aerosolized CpG-ODN against N202.1A mammary tumor cells

The efficacy of CpG-ODN aerosolization *versus* i.p. administration in controlling the growth of experimental lung metastases was evaluated in mice bearing the immunogenic N202.1A tumor, a mammary carcinoma overexpressing the rat neu oncogene. FVB mice were injected i.v. with 3×10^5 N202.1A cells and, 72 hr later, treated at 72–96 hr intervals for 3 weeks with aerosolized 5 ml saline containing 1.5 or 2.5 mg CpG-ODN (to treat up to 10 mice in the same aerosol box) or with 20 μg/mouse CpG-ODN administered i.p. (in 200 μl saline). At 5 weeks after treatment, the number of lung colonies was significantly lower in mice treated with 1.5 or 2.5 mg CpG-ODN aerosolization ($p < 0.001$ 1.5 mg CpG-ODN *vs.* control; $p < 0.05$ 2.5 mg CpG-ODN *vs.* i.p.), but not in mice treated i.p. with CpG-ODN, as compared to controls (Fig. 2*a*). Each i.p. CpG-ODN injection, but not aerosolized CpG-ODN, induced transient weight loss (about 1 g) in mice (data not shown).

To compare the immune effector cells infiltrations induced in tumors by the TLR agonist through the two administration routes, the experiment above was repeated and lung infiltration of CD45+ cells was evaluated by flow cytometry after enzymatic digestion of lung tissue at day 15 after tumor cell injection. Analysis of the FCSlowSSClow fraction (Fig. 2*b*) revealed a significantly increased percentage of CD3+CD4+ T cells but no modulation of NK cells (DX5+CD3-) in the lung of mice receiving aerosolized CpG-ODN as compared to untreated N202.1A tumor-bearing mice, while i.p. administration induced a significant increase of NK cells but did not modulate the percentage of T cells (CD3+CD4+ or CD3+CD8+). Thus, locally administered CpG-ODN was more effective in promoting an expansion of CD4+ T cells in lungs bearing an immunogenic tumor, whereas i.p. administration preferentially expanded NK cells.

Anti-tumor activity of aerosol CpG-ODN treatment in B16 melanoma tumors

As most primary lung tumors and frequently lung metastases derived from other tumor histotypes in humans are only weakly immunogenic, we compared the efficacy of the two administration routes against B16 murine melanoma, in which protection is mediated by NK cells.[30–32] Mice were injected i.v. with 5×10^5 B16 cells and treated with 1.5 mg

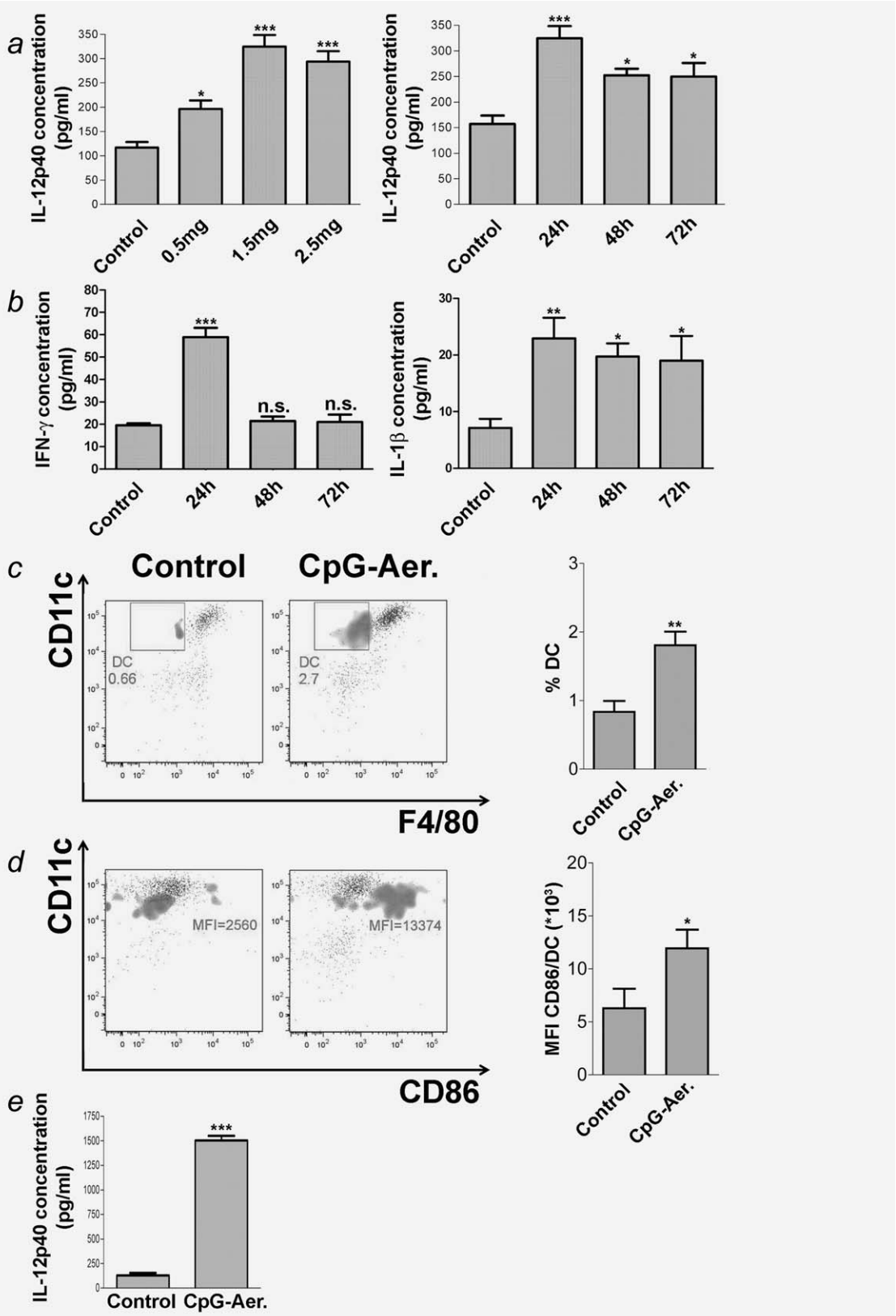

Figure 1. Immune effects of aerosolized CpG-ODN in BAL fluid. (*a*) IL-12p40 levels evaluated by ELISA in BAL of mice at 24 hr after aerosol with CpG-ODN at different concentrations (left) and at different times after a single treatment with 1.5 mg of CpG-ODN (right). (*b*) IFN-γ and IL-1β levels evaluated by proteomic analysis in BAL collected 24, 48 and 72 hr after a single CpG-ODN aerosolization (1.5 mg). All values (pg/ml) are expressed as mean ± SE (5–6 mice/group). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ *versus* control, by one-way ANOVA followed by Dunnet's post-test. (*c*) A representative flow cytometric analysis of DC (identified as CD11c+F4/80- cells among CD45+ cells after lymphocyte exclusion) in BAL after 4 CpG-ODN (1.5 mg) or saline aerosol treatments at intervals of 72–96 hr (left). Histogram on the right shows the frequency of DC in 8–10 mice per group. **$p < 0.01$, *$p < 0.05$ *versus* control, by Student's *t* test. (*d*) A representative flow cytometric analysis of CD86 expression level, represented as mean fluorescence intensity (MFI) performed in the DC gate (as described in Panel *c*) (left). Histogram on the right shows the CD86 MFI in the DC gate in 8–10 mice per group. **$p < 0.01$, *$p < 0.05$ *versus* control, by Student's *t* test. (*e*) IL-12p40 levels in BAL collected at 24 hr after the last of four treatments at 72–96 hr intervals with aerosolized CpG-ODN (1.5 mg). Values (pg/ml) are expressed as mean ± SE (5–6 mice/group). ***$p < 0.001$ *versus* control, by Student's *t* test.

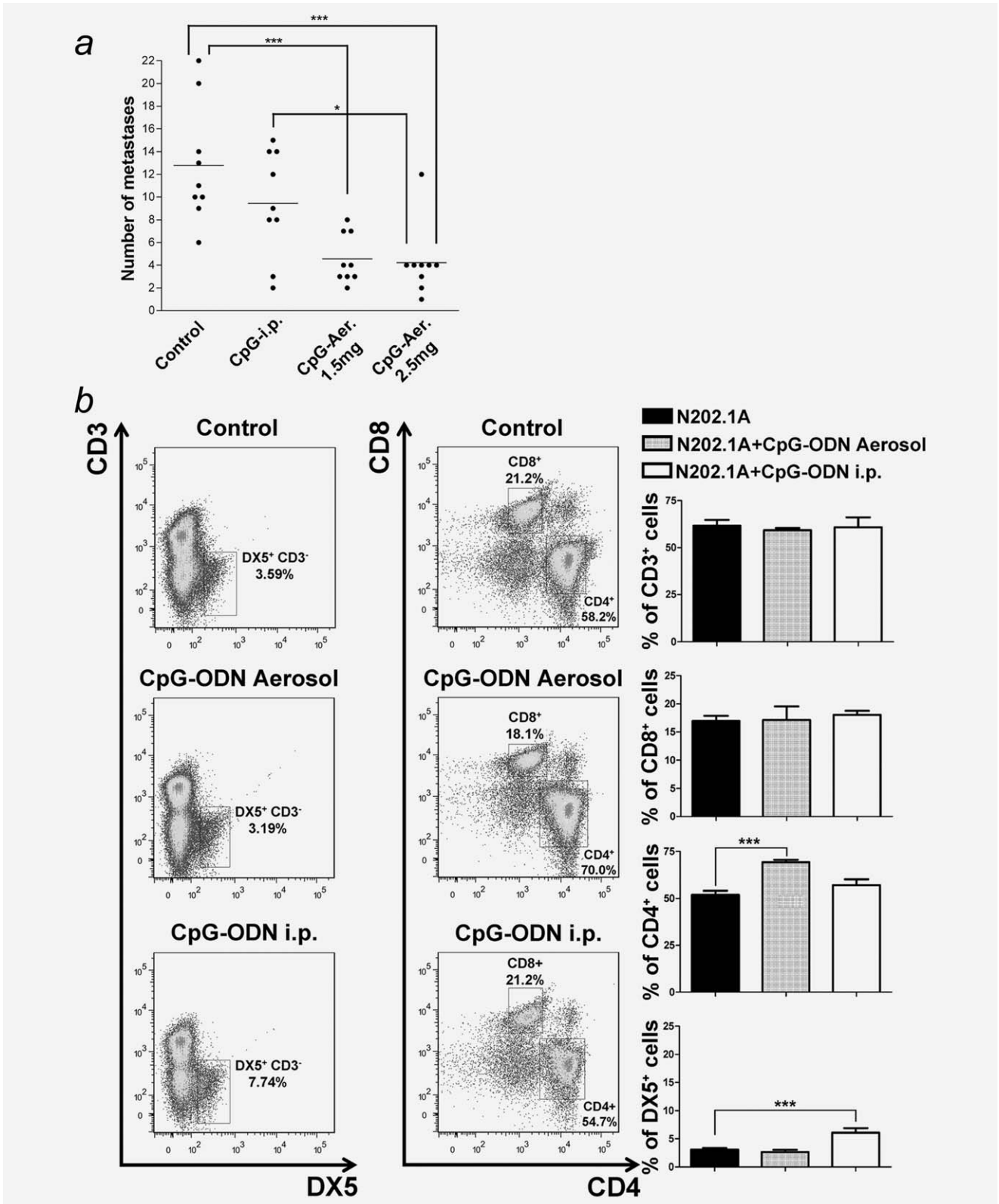

Figure 2. Effect of aerosol or intraperitoneal administration of CpG-ODN on N202.1A experimental lung metastases. (*a*) Number of macroscopic lung metastases at 5 weeks after i.v. injection of N202.1A mammary carcinoma cells in mice untreated or treated with CpG-ODN i.p. or by aerosol (9 mice/group). *$p < 0.05$; ***$p < 0.001$ by one-way ANOVA followed by Tukey's post-test. (*b*) A representative flow cytometric analysis of immune infiltrate of lungs of mice at 2 weeks after i.v. injection with N202.1A tumor cells untreated or treated with CpG-ODN aerosol or i.p. NK cells were identified as DX5+CD3-, while CD4+ and CD8+ T cells were identified in CD3+ gated cells (among CD45+ FSClowSSClow-gated cells). Histograms show the frequency of different populations in 5 mice per group (mean ± SE). ***$p < 0.001$ by one-way ANOVA followed by Dunnet's post-test.

aerosolized or i.p.-administered CpG-ODN (20 μg/mouse) at 72–96 hr intervals for 3 weeks, beginning 72 hr after tumor cell injection. A third group of mice was left untreated as a control for lung tumor colonization. In contrast with the N202 tumor model, mice treated with CpG-ODN aerosol showed no significant reduction in the number of lung metastases at 5 weeks, while i.p. CpG-ODN administration induced significant protection ($p < 0.0001$ CpG-ODN i.p. *vs.* control) (Fig. 3*a*). Flow cytometric analysis of CD45+ immune effector cells in treated or untreated mice after enzymatic digestion of lung tissues at day 15 after tumor injection indicated that neither aerosol nor i.p. administration expanded CD3+CD4+ and CD3+CD8+ T cells in this tumor model, whereas i.p., but not aerosol CpG-ODN, treatment induced a strong increase in the percentage of NK cells (Fig. 3*b*). The increased percentage of NK cells in the lung induced by systemic treatment did not appear to reflect specific tumor-induced recruitment but rather an increased number of CpG-induced circulating NK cells, because a similar expansion of DX5+CD3- cells was detected in lung as well as in spleen and blood of tumor-free mice after i.p. injection of CpG-ODN (23.7% ± 1.7% in i.p.-treated *vs.* 12.5% ± 0.9% in untreated mice in the lung; 4.8% ± 0.2% and 5.3% ± 0.5 % in i.p.-treated *vs.* 3.4% ± 0.1 % and 2.7% ± 0.3 % in untreated mice in the spleen and blood, respectively; 4 mice/group).

Alveolar macrophages as suppressive immune effectors in lung infiltrate of metastases-bearing mice

With progressive tumor growth, resident pulmonary alveolar macrophages, which are the most abundant inflammatory cells in the lungs, frequently shift their polarization to the M2 phenotype and exert suppressive activity on T and NK cells.[33–36] Double immunofluorescence to detect the presence of macrophages (CD68+) secreting IL-10, a marker of the M2 phenotype, in lungs of mice injected i.v. with B16 or N202.1A tumor cells revealed a high number of CD68+ cells secreting IL-10 in B16 tumor-bearing mice, whereas only low number of CD68+/IL-10+ double-positive cells was detected in the lungs of N202.1A tumor-bearing mice (Fig. 4). Thus, the inability of aerosol CpG-ODN to induce effective immune activation in B16 tumor-bearing lungs might be due to a strong immunosuppressive activity established by alveolar macrophages in this tumor microenvironment, raising the possibility that depletion of macrophages might allow NK expansion induced by aerosol CpG-ODN in this tumor model. Having determined empirically that a single i.t. injection of clodronate encapsulated liposomes greatly reduces the percentage of alveolar macrophages (% of CD45+CD11c+CD11b- in digested lungs: 2.66±1.3 in Cl_2MDP liposome-treated *versus* 11.17±2.3 in liposome-treated mice evaluated 72 hr after injection), consistent with previous data,[37] we tested the effect of macrophage depletion on aerosol CpG-ODN-induced NK cell expansion in four groups of mice (5 mice/group) injected i.v. with 5×10^5 B16 cells and, after 96 hr, injected i.t. with 100 μl of Cl_2MDP-liposome (three groups) or 100 μl of control liposome (the fourth group). After 24 hr, two groups of mice given Cl_2MDP liposomes started the treatment with aerosol (1.5 mg) or i.p. (20 μg) CpG-ODN for 4 days/week for 2 weeks, while the third and fourth groups treated with Cl_2MDP liposome and control liposome, respectively, received no CpG-ODN. Analysis of the phenotype and composition of the immune cell infiltrate in the lung at 24 hr after the last CpG-ODN treatment (Fig. 5*a*) indicated that in the absence of resident macrophages, aerosol CpG-ODN treatment did indeed induce significant NK cell expansion in B16 tumor-bearing lungs. In these lungs, the increased percentage of DX5+CD3- cells gated in the CD45+ population corresponded to a significant decrease of the percentage of CD3+ cells. Mice treated with clodronate alone revealed a reduced percentage of alveolar macrophages without modification in the percentage of NK and CD3+ cells as compared to mice treated with control liposomes.

Anti-tumor activity of aerosol CpG-ODN treatment against B16 melanoma cells in clodronate-treated mice

To determine whether the changes in cellular immune lung infiltrates induced by CpG-ODN aerosol in clodronate-treated B16 tumor-bearing mice would lead to improved anti-tumor activity, six groups of mice were injected i.v. with B16 cells and, after 96 hr, injected i.t. with Cl_2MDP liposomes (three groups) or control liposome (three groups) but started on treatment with CpG-ODN aerosol (one group Cl_2MDP liposome-treated and one group liposome-treated) or i.p. (one group Cl_2MDP liposome-treated and one group liposome-treated) at day 5 (120 hr) after tumor injection to allow resident macrophage depletion before treatment. Treatment was continued for 4 days/week for 3 weeks. The remaining two groups of Cl_2MDP liposome- and control liposome-treated mice received no CpG-ODN. Aerosol CpG therapy in macrophage-depleted mice induced a significant reduction in the number of lung metastases, as observed 5 weeks after tumor injection (Fig. 5*b*), whereas macrophage depletion did not modify the efficacy of i.p. treatment. Moreover, clodronate-induced macrophage depletion *per se* did not reduce tumor growth, as the number of lung metastases in Cl_2MDP liposome- *versus* control liposome-treated mice did not differ significantly. These data indicate that depletion of resident macrophages in tumor-bearing mice allows CpG aerosol therapy to stimulate a local immune response that confers significant protection from lung metastases.

As Ly6G+ granulocytic polymorphonuclear neutrophil subsets of myeloid-derived suppressor cells (PMN-MDSC) reportedly impair NK cell development specifically[38–40] and as cross-talk between tumor-associated macrophages and MDSCs has been shown to sustain and exacerbate immune suppression in the tumor microenvironment,[41] we tested whether this population was also involved in preventing NK cell expansion induced by CpG-ODN aerosol. Four groups of

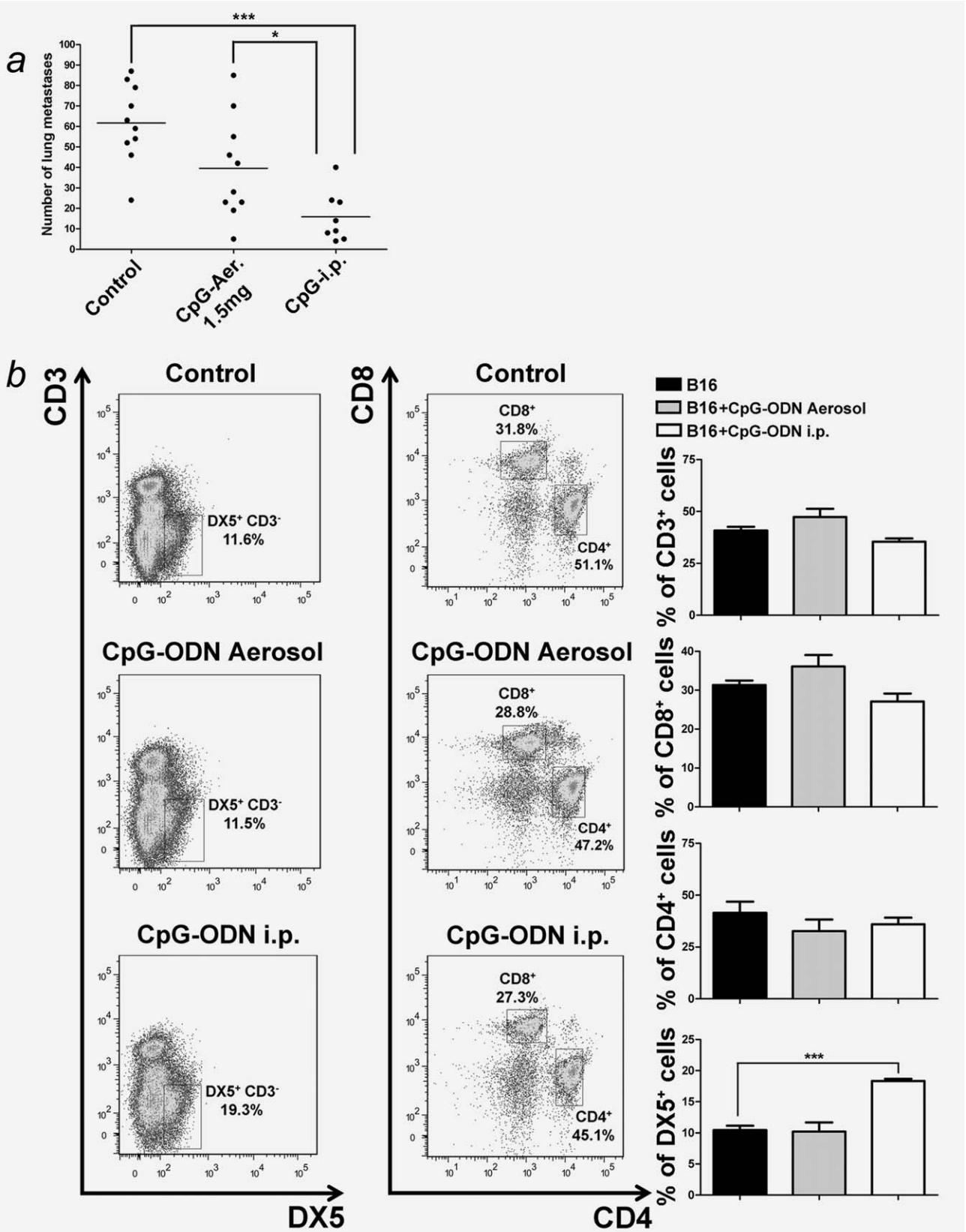

Figure 3. Effect of aerosol or intraperitoneal administration of CpG-ODN against B16 experimental lung metastases. (*a*) Number of macroscopic lung metastases at 5 weeks after i.v. injection of B16 melanoma cells in mice untreated (10 mice) or treated with CpG-ODN aerosol (10 mice) or i.p. (8 mice). *$p < 0.05$; ***$p < 0.001$ by one-way ANOVA followed by Tukey's post-test. (*b*) A representative flow cytometric analysis of immune infiltrate of lungs of mice at 2 weeks after i.v. injection with B16 tumor cells untreated or treated with CpG-ODN aerosol or i.p. NK cells were identified as DX5+CD3-, while CD4+ and CD8+ T cells were identified in CD3+ gated cells (among CD45+ FSClowSSClow-gated cells). Histograms on the right show the frequency of different populations in 5 mice per group (mean ± SE). ***$p < 0.001$ by one-way ANOVA followed by Dunnet's post-test.

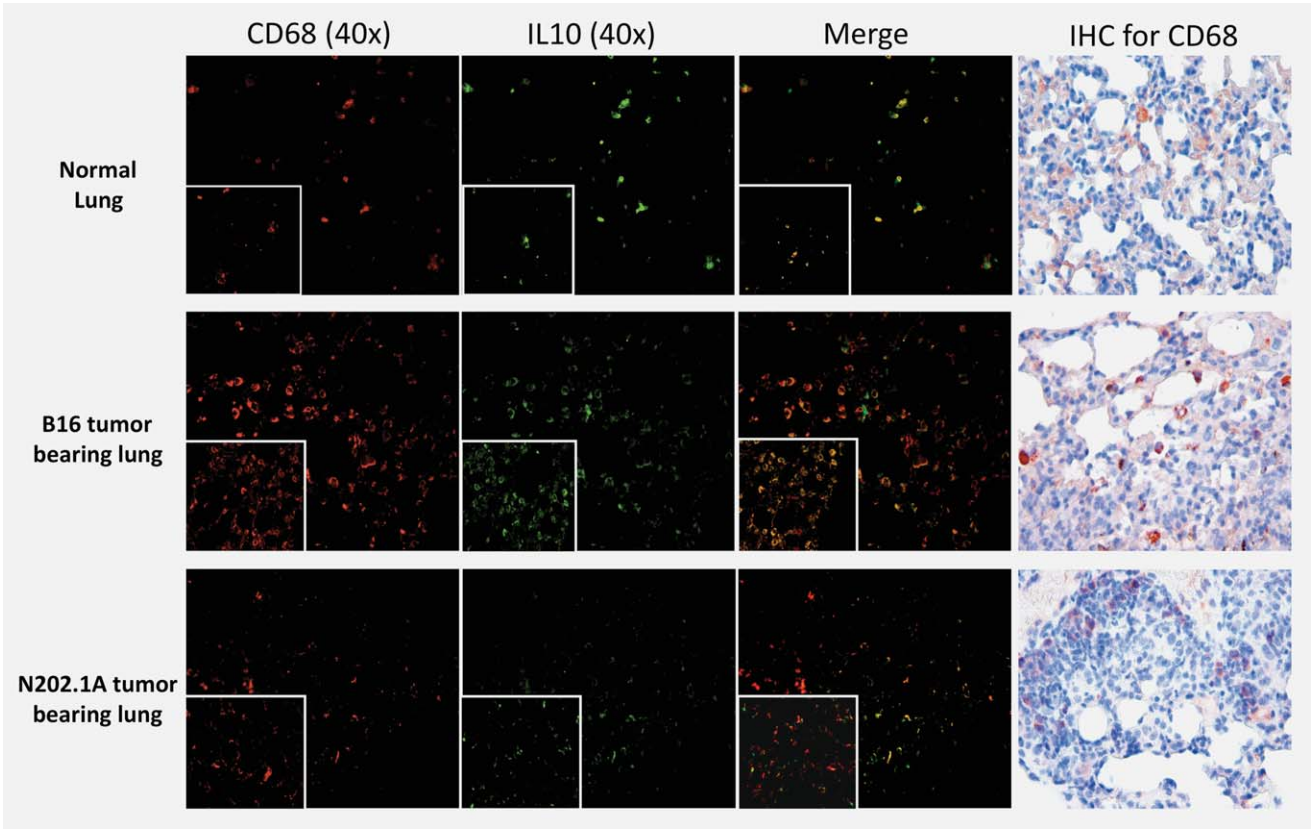

Figure 4. Analysis of IL-10-secreting macrophages in tumor-bearing lungs. Immunofluorescence analysis of sections of lung tissue collected 4 weeks after i.v. injection of B16 melanoma cells or N202.1A mammary carcinoma cells and double-stained for CD68 (red) and IL-10 (green). Representative images show single and double (merged) staining of formalin-fixed, paraffin-embedded samples. Immunohistochemical staining shows CD68+ cells populating alveolar spaces of normal lungs and lungs with either B16 or N202.1A tumor metastases. Original magnification ×400, ×200 inset magnification.

mice (5 mice/group) were injected with B16 cells as above and, 96 hr later, treated every 72–96 hr with the Ly6G-specific mAb (1A8), which recognizes and depletes Ly6Ghigh neutrophilic populations, or with isotype control. At 24 hr after the first mAb treatment, two groups of mice started the treatment with aerosolized CpG-ODN (1.5 mg) for 4 days/week for 2 weeks, while the other two groups of antibody-treated mice received no CpG-ODN. 1A8 mAb treatment induced a strong depletion of granulocytic MDSC, identified as Ly6GhighCD11b+ cells not expressing or expressing low levels of the Ly6C marker, but in the lungs of these depleted mice, CpG-ODN aerosol therapy induced only a slight expansion of NK cells (Supporting Information Fig. 1). These results suggest that granulocytic MDSC do not play a major role in macrophage-induced immunosuppression.

Discussion

Our study to explore aerosol administration as a promising route for delivery of CpG-ODN to the murine lung showed that aerosolized CpG-ODN reaches the bronchoalveolar space and can activate a local immune response, as indicated by the increased levels of IL-12p40, IFN-γ and IL-1β and the recruitment and maturation of DC in BAL fluid. Consistent with previous findings that alveolar macrophages respond to CpG-ODN with production of IL-12p40 but not IL-12p70,[13] we detected no production of IL-12p70 in BAL fluids, which contain alveolar macrophages almost exclusively. No signs of apparent toxicity, evaluated as body weight loss and as histological changes in lung architecture, were observed after repeated CpG-ODN aerosol administration, in contrast with the adverse effects reported in mice receiving CpG-ODN i.n. or i.t.[27–29]

The anti-tumor activity of aerosol-delivered CpG-ODN was evaluated in two murine tumor models with different characteristics and behavior: the mammary carcinoma N202.1A is highly immunogenic due to overexpression of the heterologous rat neu oncogene,[25] while the B16 melanoma is poorly immunogenic and reportedly expresses very low levels of MHC I molecules.[42] These two tumors also differ in their sensitivity to immune effectors, with the neu-expressing mouse mammary tumor sensitive to cytotoxic activity of T but not NK cells,[43] while NK cells are required to counteract the growth of B16 melanomas.[30–32] We observed that while N202.1A tumors in the lung induced a low level of CD68+ macrophages secreting IL-10, B16 melanoma cells strongly promoted the presence of double-stained CD68+IL-10+

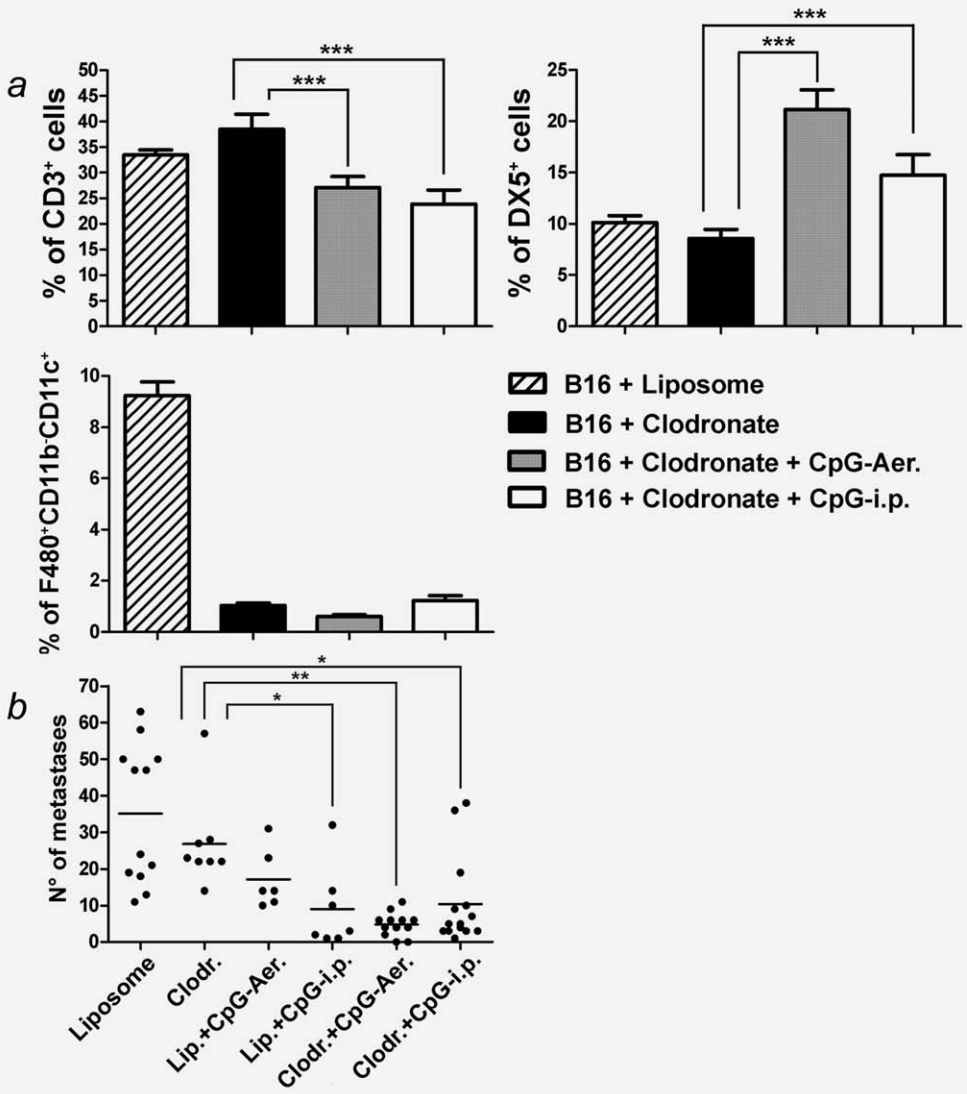

Figure 5. Effect of clodronate-induced macrophage depletion on aerosol or intraperitoneal CpG-ODN treatment of B16 lung metastases-bearing mice. (*a*) Frequency of CD3+ T cells (among CD45+ FSClowSSClow-gated cells), NK cells (DX5+CD3- among CD45+ FSClowSSClow-gated cells) and alveolar macrophages (F4/80+ CD11b- CD11c+ among CD45+ gated cells) evaluated by flow cytometric analysis of immune infiltrate of lungs of mice at 2 weeks after i.v. injection with B16 tumor cells and i.t. treatment 96 hr later with clodronate encapsulated liposomes or PBS-liposomes. At 24 hr after liposome treatment, two groups of clodronate-treated mice started treatment with CpG-ODN i.p. or aerosol. Histograms represent pooled data from 5 mice per group (mean ± SE). ***$p < 0.001$ by one-way ANOVA followed by Dunnet's post-test. (*b*) Number of macroscopic lung metastases at 5 weeks after i.v. injection of B16 melanoma cells in mice injected i.t with PBS-liposomes alone (12 mice), clodronate encapsulated liposomes alone (8 mice), PBS-liposomes followed by aerosol (6 mice) or i.p. (7 mice) CpG-ODN treatment, or clodronate encapsulated liposomes followed by aerosol (12 mice) or i.p. (14 mice) CpG-ODN treatment. *$p < 0.05$; ***$p < 0.001$ by one-way ANOVA followed by Tukey's post-test.

cells, suggesting the potential of the latter model to induce an immunosuppressive microenvironment. Accordingly, Gil-Bernabè *et al.*[44] have recently observed in lungs from mice injected with B16 melanoma cells the selective recruitment of CD68+ macrophages essential for tumor cell survival. These observations are consistent with studies showing that alveolar macrophages in the presence of some growing tumors become immunosuppressive,[33,35] *i.e.*, develop M2 tumor-associated macrophages, which play a role in the progression and prognosis of lung cancer.[45,46] Depending on their relative importance, any or all of these differences might underlie the different results observed in the two models.

In the immunogenic N202.1A model, aerosol-delivered CpG-ODN induced the preferential expansion of CD4+ cells and was more efficacious than i.p. administration in control lung metastases, suggesting that the local addition of a TLR agonist might help the immune system recognize and eliminate tumor. Our preliminary experiments demonstrating a complete cure of N202.1A lung metastases in 5 of 10 mice treated with aerosol 15 mg CpG-ODN/5 ml

saline without reduction of weight suggest the promise of increasing the dose of aerosolized CpG-ODN to achieve complete protection in this immunogenic model. In contrast, i.p. delivery of CpG-ODN in the B16 melanoma model stimulated a strong expansion of NK cells and induced significant protection against lung metastases, while CpG aerosol treatment failed to modify the number of NK cells in lung and did not confer significant protection. Depletion of lung macrophages by liposome encapsulated clodronate, a bisphosphonate compound,[47] allowed aerosolized CpG-ODN to expand NK cells and reduce the number of lung metastases. Aerosol inhalation to deliver Cl_2MDP-liposomes to the lung has been recently reported,[48] raising the possibility of aerosolized bisphosphonates in clinical use. In contrast, depletion of the MDSC granulocytic component by anti-Ly6G antibody induced only a slight increase in NK cell expansion, excluding major involvement of this component in immunosuppression. Thus, other direct or indirect mechanisms, such as those induced by the production of suppressive cytokines as well as prostaglandin E, hydrogen peroxide and superoxide by alveolar macrophages that support the maintenance of the suppressive microenvironment[33] might be involved in inhibition of CpG local activity.

Several studies have reported the superior anti-tumor effect of CpG administered directly rather than systemically into the tumor in both immunogenic or nonimmunogenic tumor models, since CpG-ODN can activate both innate and adaptive immune responses at the tumor site.[6–9] Our results indicate that this is not true for tumors growing in the lung, where both tumor immunogenicity and the tumor-induced immunosuppressive environment are critical factors in the success of CpG therapy. Thus, different administration routes might be required for different tumors.

The composition of immune cells localized to the lung likely reflects the degree to which the immune system is subverted by the tumors, consistent with the reported prognostic value of characterization of immune infiltrate in the lung in non-small cell lung carcinoma and in lung adenocarcinoma.[49,50] Evaluation of the patient lung immune status through analysis of BAL composition[50] might provide a noninvasive means of routinely sampling the immune environment of the lung to define the best immunotherapeutic strategy.

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