Plasmidic CpG sequences induce tumor microenvironment modifications in a rat liver metastasis model

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Abstract. Bacterial DNA contains unmethylated cytosine-phosphate-guanine (CpG) motifs which are recognized by mammalian immune cells as a danger signal indicating an infection. These immunostimulatory properties led to the use of oligodeoxynucleotides bearing CpG motifs (CpG-ODN) for cancer treatment in preclinical and clinical studies. Although naked DNA administration presently represents 18% of the gene therapy clinical trials worldwide, most of the work regarding the effects of unmethylated CpG sequences was performed using CpG-ODN. In the present study, we analyzed early induced tumor microenvironment modifications in a rat liver metastasis model after intratumoral injection of a plasmid used in suicide gene therapy. We first showed that plasmidic CpG motifs were active, i.e. able to induce IFN-γ secretion by rat splenocytes. Then, we compared tumor-infiltrating immune cells 24 h after injection of native or Sss*I*-treated plasmid, in which immunostimulatory CpG motifs have been inactivated by methylation. The presence of active plasmidic CpG sequences within the tumor was associated with a decrease in the number of tumor-infiltrating conventional dendritic cells and an upregulation of the CCR7 chemokine receptor responsible for lymph node homing. We also observed an increase in plasmacytoid dendritic cells and natural killer cell infiltration within the tumors as well as an increased mRNA expression of three cytokines/chemokines (IL-1ß, IL-10 and IL-18). These data suggest that, although suicide plasmid injection without prodrug treatment is not sufficient to observe a therapeutic effect, the presence of plasmidic CpG motifs within the tumor induces the recruitment and activation of the immune cells involved in antitumor response. These early cellular and molecular events should facilitate the induction of the immune response against tumor antigens released after *in situ* drug production.

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Introduction

Colon carcinoma remains a leading cause of cancer death due to the development of liver metastases. As surgery, which constitutes the only curative treatment, can be performed in only 10% of metastatic patients (1,2), an alternative treatment such as gene therapy needs to be developed. In this context, we developed a suicide gene therapy approach using the bacterial cytosine deaminase (CD) gene to convert the non-toxic antifungal agent 5-fluorocytosine (5-FC) into the widely used chemotherapeutic drug 5-fluorouracil (5-FU) (3).

We previously demonstrated, in a syngeneic rat bifocal liver metastasis model, that injection of a CD-expressing plasmid in one of the tumors, followed by 5-FC treatment of the animals, resulted in the regression of both lesions due to the triggering of a strong distant bystander effect (4). Although plasmid DNA injection without prodrug treatment was not sufficient to induce a therapeutic effect in this model (4), plasmid DNA could be involved in the potent antitumoral response observed via the presence of unmethylated CpG sequences in the plasmid backbone. Indeed, plasmid DNA, as bacterial DNA, contains unmethylated cytosine-phosphate-guanine (CpG) sequences that are considered immunostimulatory. CpG motifs are 4 times more frequent in bacterial than in mammalian DNA, and they are generally methylated in mammals and unmethylated in bacteria (5). These differences lead the immune system to recognize unmethylated CpG sequences as a 'danger signal' indicating a potential pathogen infection (5). In immune cells, recognition of these motifs mediated by the Toll-like receptor 9 (TLR9) (6) is followed by downstream signaling events leading to the activation of transcription and translation of target genes (7). As a

consequence, bacterial CpG motifs trigger pleiotropic effects in immune cells such as proliferation, activation and cytokine/chemokine secretion (5). An intratumoral injection of CpG oligonucleotides (CpG-ODN), which mimic bacterial DNA, was shown to exert antitumoral effects in various experimental tumor models (8-11). This therapeutic effect involving $CD8^+$ T cells or natural killer (NK) cell activation can also induce the recruitment of macrophages or dendritic cells (DCs) within the tumor (9,10) or in draining lymph nodes (11).

As most of the work regarding the effects of unmethylated CpG sequences was performed using CpG-ODN and naked DNA administration presently represents 18% of the clinical trials worldwide (Wiley website 2007; http://www.wiley.co.uk/genmed/clinical/), we investigated the plasmidic CpG effects *in vivo*. To this aim and towards prospective future clinical trials, we analyzed the cellular and molecular events early induced *in vivo* after intratumoral injection of the CD-expressing plasmid with or without functional CpG sequences in a metastatic colon carcinoma model.

Materials and methods

Plasmid and CpG-ODN. The pCDßgeo plasmid contains the cytosine deaminase (CD) gene under the control of the CMV promoter and a neo-lacZ fusion gene located downstream from the PGK promoter (3). Plasmid DNA was prepared using the Qiagen EndoFree Plasmid Mega Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions (endotoxins <0.1 EU/μg of DNA). Phosphorothioate-modified oligonucleotides (ODNs) were purchased from Proligo (Paris, France). The following sequences were used (the underlined letters indicate the CpG motifs or the CpC motifs of the negative control ODN). CpG-ODN 1826, 5'-TCCATGA<u>CG</u>TTCCTGA<u>CG</u>TT-3' and CpC-ODN, 5'-TCCATGA<u>CC</u>TTCCTGA<u>CC</u>TT-3'.

Plasmid methylation. The pCDßgeo plasmid (200 μg in a 1-ml final volume) was methylated with Sss*I* CpG methylase (New England Biolabs, Ozyme, St Quentin en Yvelines, France) at 2 U enzyme/μg of DNA for 4 h at 37˚C, according to the manufacturer's instructions. DNA was then extracted with phenol/chloroform, precipitated with ethanol, and washed with 70% ethanol. Methylated plasmid (Met-P) was quantified, and the efficiency of methylation was confirmed by BstU*I*digestion. The native pCDßgeo plasmid (P) was subjected to the same treatment except that Sss*I*CpG methylase was omitted in the reaction mix.

Cell culture. DHD/K12/PROb (PROb) cells constitute a colon carcinoma cell line originating from a chemically induced colon cancer in BDIX rats (12). These cells are poorly immunogenic and induce progressive and metastatic tumors in syngeneic hosts. PROb cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Dutscher, Brumath, France), 50,000 UI/l penicillin, 100 μmol/l streptomycin, 2 mmol/l L-glutamine at 37˚C and in 8% CO_2. For generation of liver tumors, cells were trypsinized, washed and re-suspended at $15x10^6$ cells/ml in cold phosphate-buffered saline. For measurement of *in vitro* cytokine production, rat splenocytes (10^6 cells in 200 μl/well) were cultured in standard 48-well plates in RPMI-1640 (Cambrex, Vervier, Belgium) supplemented with 10% fetal calf serum, 50,000 UI/l penicillin, 100 μmol/l streptomycin, 2 mmol/l L-glutamine at 37˚C in 5% CO_2 and stimulated with ODNs (one dose of 0.15 μg/well), or plasmids (three doses of 3 μg/well) or treated with PBS (5 μl/well). Supernatants were collected after 24, 30 or 48 h and stored at -80˚C until ELISA analysis.

Measurement of IFN-γ production. Supernatants from spleen cell cultures described above were analyzed by ELISA according to the manufacturer's instructions (Biosource, Cliniscience, Montrouge) 24, 30 and 48 h after stimulation by DNA.

Experimental protocol. For all of the experiments, we used adult (9 week-old) BDIX male rats weighing 200-250 g (Charles River, L'arbresle, France). All the surgical procedures and the care given to the animals were in accordance with institutional guidelines. After anesthetization of the rats, the livers were surgically exposed, and $1.5x10^6$ PROb tumor cells (100 μl) were injected under the Glisson's capsule using a 27-gauge needle to generate experimental liver metastases. Fifteen days later, the rats underwent surgery, the livers were exposed, and 100 μl of plasmid DNA solution (P or Met-P, 1 mg/ml) was directly injected into the tumors by using a 27-gauge needle. Twelve (gene expression analysis) or 24 h (immune cell recruitment analysis) after intratumoral DNA injections, the rats were anesthetized before being sacrificed, and the tumors were removed for analysis. Freshly prepared rat splenocytes without erythrocytes were used for measurement of *in vitro* IFN-γ production. Briefly, rats were sacrificed, and single-cell suspensions were prepared by mechanical dissociation of spleen and erythrocyte lysis by incubating the cells for 10 min at 4˚C in buffer (155 mM NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA, pH 7.4).

Isolation of tumor-infiltrating immune cells. Tumor specimens were pooled (6-12 tumors per experimental group), sliced into small pieces, and digested in DMEM supplemented with 3% SVF, 2 mg/ml collagenase A, 60 μg/ml DNase I (Roche Molecular Biochemicals, Mannheim, Allemagne) for 2x 45 min at 37˚C with gentle agitation. The resulting cell suspensions were filtered through a nylon membrane and centrifuged for 5 min at 4˚C (400 g). The cells were washed in PBS containing 5 μg/ml DNase I and 3% SVF, centrifuged for 5 min at 4˚C (400 g), re-suspended in cold PBS containing 50 μM EDTA and 3% SVF, and counted. For flow cytometry analysis, a low-density cell enrichment was performed using Optiprep™ solution, 1,059 g/l, pH 7.2 (Nyegaard Diagnostics, Oslo, Norway) for 10 min at 4˚C (400 g). The low-density cell fraction was successively depleted in γδ T lymphocytes and enriched in $\gamma\delta^-$, $OX62^+$, conventional dendritic cells using, respectively, mouse anti-rat γδ TCR and anti-CD103 magnetic bead-coated mAbs according to the manufacturer's instructions (Miltenyi Biotec, Paris, France).

Flow cytometry. The number of conventional activated DCs, plasmacytoid dendritic cells (pDCs), and NK cells in the total tumor cell suspensions was determined by flow cytometry

analysis following triple stainings with different monoclonal antibodies. Mouse mAbs against γδ TCR (V65 clone, biotin-conjugated), CD86 activation marker (24F clone, biotin-conjugated) and CD4 (OX-35 clone, FITC-conjugated) were purchased from BD Biosciences. Mouse mAbs against CD103 (CL083 clone, OX-62, FITC-conjugated), NKR-P1 (CL055 clone, FITC-conjugated) and MHC class II (CL011 clone, RT1.D, PE-conjugated) were purchased from Cedarlane. Tricolor avidin was purchased from Caltag (Burlingame, CA). Mouse IgG1 and IgG2a were used as isotype controls. Before specific staining, cell surface Fc receptors were blocked by incubation with purified hyper-immune rat serum (processed in-lab). All the staining steps were performed at 0-4˚C in PBS containing 5 mM EDTA and 3% FCS. Analysis was performed on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA) at the Flow Cytometry Laboratories of IFR50 (Faculty of Medicine, Nice, France) using Cell Quest Pro software (Becton Dickinson).

Immunohistologic analysis. Six-micrometer cryosections of tumors were fixed in acetone for 5 min at -20˚C. Endogen biotin was blocked with 5% goat serum and 10% streptavidin (Vector Labs, AbCys, Paris, France) in PBS buffer for 30 min at room temperature, and endogenous peroxidase activity was quenched using 3% H_2O_2 for 10 min at room temperature. NK cells were detected by overnight incubation at 4˚C with mouse mAb against NK cells (NKR-P1A, 10-78, Pharmingen, BD Biosciences, Le Pont de Claix, France) in antibody diluent and 10% biotin (Vector Labs). The isotype antibody (mouse IgG, I-2000, Vector Labs, Burlingame, CA) was used as a negative control. After washing in phosphate-buffered saline, slides were incubated with biotinylated goat anti-mouse immunoglobulins (BD Biosciences, Le Pont de Claix, France) for 1 h at room temperature, washed, and subsequently incubated with streptavidin-horseradish peroxidase (HRP) conjugate (Pharmingen). After washing, positive cells were visualized using HRP substrate (VIP, Vector Labs). The slides were then counterstained with hematoxylin and treated with mounting medium (VectaMount, Vector Labs) before observation.

RNA extraction. Eight tumors per condition were pooled, and low-density cell enrichment was realized using Optiprep™ solution (1,065 g/l) as previously described. Total RNA of the low-density cell fraction was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Integrity of the RNA was tested on a 1% agarose gel, and quantification was carried out by absorption at 260 nm.

RNase protection assay (RPA). RPA was performed with RiboQuant™ Multi-Probe RNase Protection Assay Kits (BD Biosciences), according to the manufacturer's instructions. Briefly, [^{32}P]-labeled antisense riboprobes were synthesized with rCK-2 or rCK-3 template set (BD Biosciences), using 2.75 mM GTP, ATP, CTP, 80 μCi [α-^{32}P]-UTP (3000 Ci/mmol, MP Biomedicals, Vannes, France) and 20 U T7 RNA polymerase. [^{32}P]-labeled antisense riboprobes were hybridized with 9 μg of total RNA of the low-density cell fraction at 56˚C overnight. After hybridization, 20 ng RNase A and 60 U RNase T1 were added to digest unhybridized RNA for 45 min at 30˚C. Proteinase K (12 μg) was then added to the sample, and the mixture was incubated at 37˚C for an additional 15 min. At the end of the incubation, the sample was extracted with phenol/chloroform and ethanol precipitated. The pellet, containing the duplex RNA hybrids was loaded onto a 5% polyacrylamide gel under denaturating conditions (7 M urea). Gels were dried and exposed to X-ray film at -70˚C for varying periods of time. Densitometry analysis was performed using Scion Image software (Scion Corp., Frederick, MD, USA).

Real-time RT-PCR analysis. Genomic DNA was removed by RNase-free DNase (Qiagen), and cDNA was synthesized using 2 μg of total RNA of the low-density cell fraction using SuperScript II Reverse Transcriptase and random hexamer primers (Invitrogen) according to the manufacturer's instructions. PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). Reactions were performed in a 20-μl volume containing 20- or 40-fold diluted cDNA, 10 μl SYBR-Green Master Mix (Applied Biosystems) and 100 nM of each primer according to the protocol provided. The oligonucleotide sequences used were as follows: ß-actin-forward, 5'-GGC CAACCGTGAAAAGATGA-3' and ß-actin-reverse, 5'-GAT GGCTACGTACATGGCTGG-3' (accession no. NM_031144, position 339-358 and 388-409); CCR7-forward, 5'-AAC GTGCTGGTGGTGGCT-3' and CCR7-reverse, 5'-CTGTGA CCTCATCTTGGCAGAA-3' (accession no. NM_199489, position 106-126 and 148-180) (Proligo). The reaction was initiated with a denaturation step of 10 min at 95˚C, followed by 40 cycles, each consisting of 15 sec at 95˚C and 1 min at 60˚C. At the end of the reaction, a progressive increase in temperature was performed to analyze putative PCR contaminants. The housekeeping gene ß-actin was used for normalization. Differences in gene expression between the different conditions were calculated using the $2^{-\Delta Ct}$ method (13).

Statistical analysis. For IFN-γ secretion, the results were expressed as the median with 95% confidence intervals (CI), and comparisons were performed using the Mann-Whitney test, which is a non-parametric, two-tailed probability test. The statistics were computed with Minitab™ Inc. V12.2 (State College, PA, USA). p values were considered to be statistically significant when <0.05.

Results and discussion

To determine whether the pCDßgeo plasmid carried functional immunostimulatory CpG sequences, we analyzed the plasmid-induced IFN-γ secretion by splenocytes. Rat spleen cells, which constitute a CpG-responsive population (5), were cultured in the presence of native plasmid (P) or Sss*I*-methylated plasmid (Met-P), in which the effect of CpG sequences had been abolished by methylation, or CpG-ODN stabilized by a phosphorothioate backbone used as a positive control. The medium of the negative control cultures was supplemented with PBS or with non-CpG-ODN (CpC). At

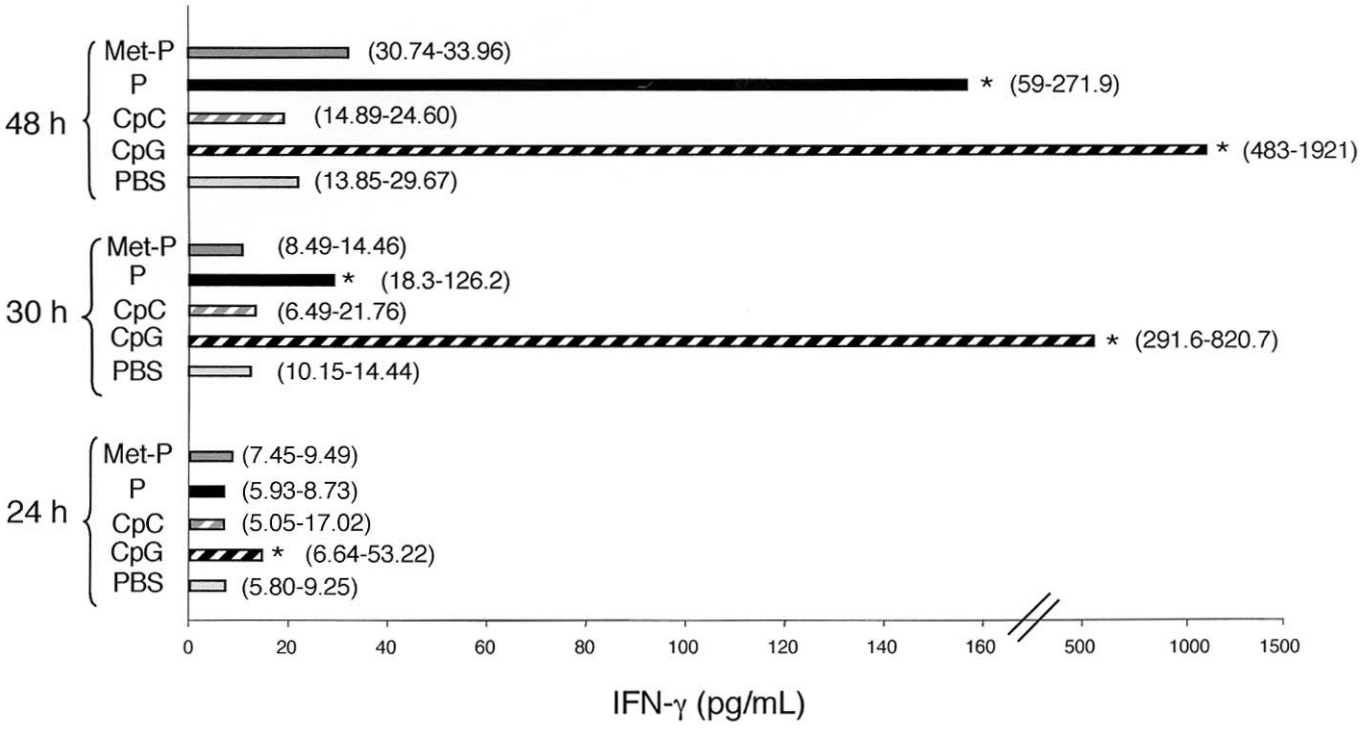

Figure 1. Analysis of IFN-γ secretion in rat spleen cells cultured in the presence of native or methylated pCDßgeo plasmid. Rat spleen cells (10^6) were cultured in the presence of CpG-ODN (CpG) or native (P) or methylated (Met-P) pCDßgeo plasmid for 24, 30 and 48 h. Cultures in the presence of PBS or CpC-ODN (CpC), devoid of CpG immunostimulatory motifs, were used as negative controls. Bars represent the median IFN-γ secretion from 4 to 8 ELISA wells in each condition with 95% confidence intervals. *p<0.05, significant difference compared to the PBS condition.

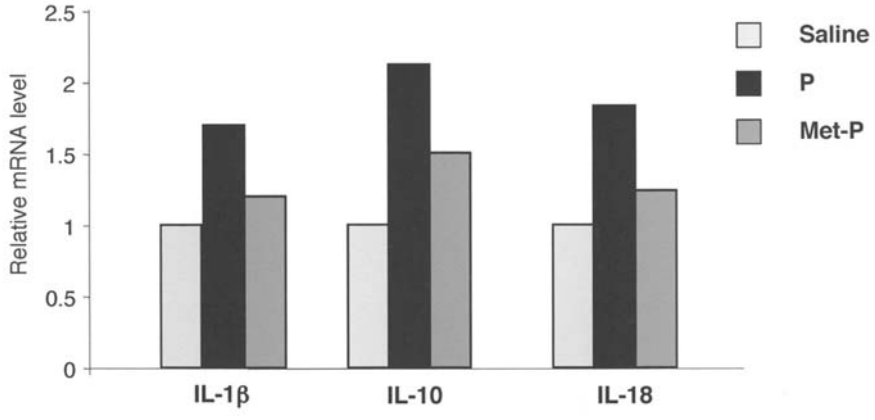

Figure 2. Analysis of cytokine/chemokine mRNA expression following plasmidic CpG intratumoral injection. The cytokine microenvironment analysis of the tumor by RNase Protection Assay. Twelve hours after saline, P or Met-P injection, 8 tumors per condition were pooled, and a population enriched in tumor-infiltrating immune cells was isolated by selection of low-density cells. Total RNA was extracted from these cells, and the Multi-Probe RNase Protection Assay was performed to determine the mRNA expression of different cytokines/chemokines. The results are expressed as the ratio of the studied gene relative to L32 which encodes a ubiquitously expressed ribosome subunit protein used as an internal control for normalization. Mean values obtained in the saline condition were assigned the value of 1 (arbitrary units).

different time points, the supernatants were collected and analyzed by ELISA. As shown in Fig. 1, culturing with CpG-ODN induced, respectively, a 2- (p=0.01), 46- (p=0.005) and 50-fold (p=0.03) increase in IFN-γ secretion after 24, 30 and 48 h, compared to PBS-treated cultures. The presence of native plasmid within the culture for 30 and 48 h induced, respectively, a 2- (p=0.005) and 7-fold (p=0.03) increase in IFN-γ production compared to PBS or methylated plasmid treatments. There was no significant difference between PBS, Met-P and CpC-ODN treatments. These results indicate that the pCDßgeo plasmid contained active CpG sequences which were able to stimulate rat immune cells.

To analyze whether the presence of plasmidic CpG sequences was associated with a modification in cytokine/chemokine microenvironment *in vivo*, experimental liver metastases were generated in rats, and P, Met-P or saline was intratumorally injected. Twelve hours post-injection, the rats were sacrificed, and the tumors were removed. Eight tumors in each experimental group were pooled and enzymatically dissociated, and a low-density cell fraction containing an

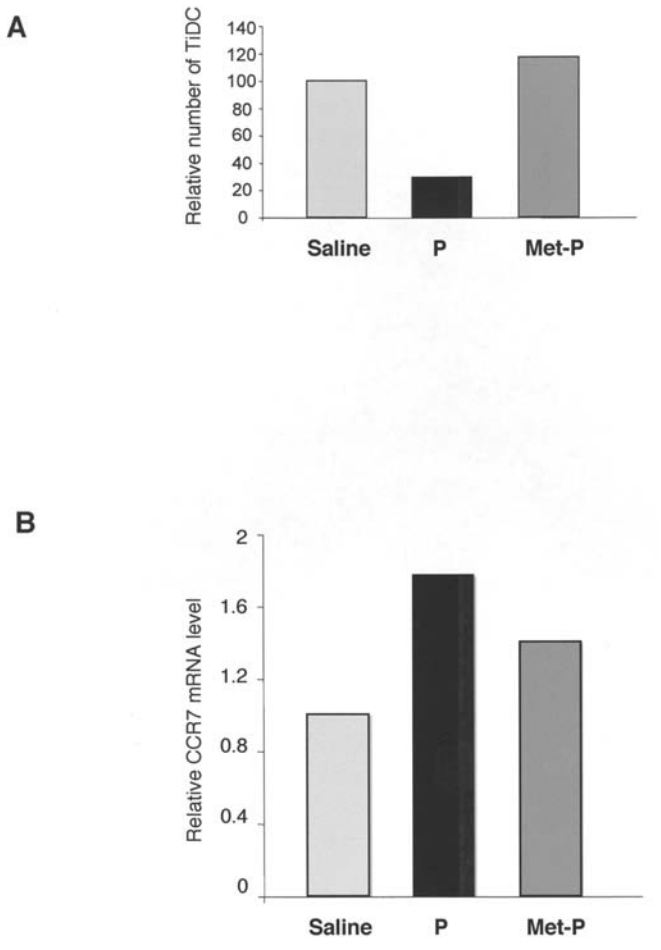

Figure 3. Analysis of tumor-infiltrating dendritic cells after plasmidic CpG injection. Twenty-four hours after saline, P or Met-P intratumoral injection, the experimental liver metastases were pooled and dissociated, and a population enriched in tumor-infiltrating immune cells was isolated by selection of low-density cells. (A) The relative number of tumor-infiltrating conventional dendritic cells (TiDC). Low-density cells were depleted in γδ T lymphocytes, and OX62$^+$ cells were then enriched using mAb-coated magnetic beads. The selected population was labeled with anti-OX62, -MHC II and -CD86 antibodies and analyzed by flow cytometry. The relative number of TiDCs in each experimental condition corresponded to the number of OX62$^+$, MHC II$^+$, CD86$^+$ conventional activated DCs normalized to the same number of total cells within the tumors. The results obtained in the saline condition were assigned the value of 100% (arbitrary units). Data are representative of three independent experiments. (B) RT-PCR analysis of CCR7 mRNA expression. Messenger RNA from the populations enriched in tumor-infiltrating immune cells were analyzed by RT-PCR using ß-actin as an internal standard 12 h after saline, P or Met-P intratumoral injection. The results obtained in the saline condition were assigned the value of 1 (arbitrary units).

enriched tumor-infiltrating immune cell population was isolated. Determination of mRNA expression for 18 cytokines/chemokines was then performed in this enriched cell population. The relative expression level of the modulated cytokines/chemokines in the different experimental groups is presented in Fig. 2. Intratumoral administration of native plasmid DNA induced a 40, 40 and 50% increase in IL-1ß, IL-10 and IL-18 mRNA expression levels, respectively, compared to Met-P-injected tumors. The expression levels for IL-12p35, IL-12p40, IFN-ß and TGF-ß3 were below the detection limit independent of the testing condition, and no significant modulation of IL-1α, IL-1-Ra, IL-6, IFN-γ, TNF-α, TGF-ß1, TGF-ß2, LT-α, LT-ß, GM-CSF and MIF mRNA expression was observed. IL-1ß and IL-18 are proinflammatory cytokines secreted as proforms requiring caspase 1 cleavage for activation (14), and we observed an increase in caspase 1 expression in these tumors (unpublished data). Although bacterial CpG motifs are essentially known as potent inducers of proinflammatory cytokines such as IL-1ß and IL-18, production of the anti-inflammatory cytokine IL-10 was recently reported following CpG stimulation (15,16). These observations suggest the existence of a two-step model where the initial TLR signaling induced pro-inflammatory cytokines, and then IL-10, thereby influencing the ensuing immune responses (15).

We further explored the influence of the plasmidic unmethylated CpG sequences within tumors *in vivo*, by analyzing the presence of conventional dendritic cells (DCs), plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells, 24 h after P, Met-P or saline intratumoral injection. We first analyzed low-density γδ$^-$, OX62$^+$, MHCII$^+$, and CD86$^+$ cells corresponding to rat activated conventional DCs (17). As shown in Fig. 3A, the presence of plasmidic CpG motifs was associated with a 70 and 85% decrease in the number of tumor-infiltrating DCs (TiDCs) compared to saline and Met-P conditions, respectively. To assess whether the decreased number of TiDCs in the native plasmid-injected tumors was related to an enhanced migratory activity towards draining lymph nodes, we analyzed mRNA expression of the CCR7 chemokine receptor in tumor-infiltrating immune cells by real-time RT-PCR. As shown in Fig. 3B, we observed a 77 and 40% increase in mRNA expression in P- compared to saline- and Met-P-injected tumors, respectively. This up-regulation of the CCR7 chemokine receptor responsible for lymph node homing (18) suggests that the presence of plasmidic CpG motifs induces dendritic cell activation and migration from the tumor to the lymph nodes. These findings are consistent with previous observations obtained after *in vivo* administration of different TLR ligands such as LPS, imiquimod or resiquimod, leading to the emigration of dendritic cells from the liver, intestine lamina propria or skin towards draining lymph nodes (19-21).

As pDCs represent a major CpG-responsive immune cell population (22), we next analyzed the number of OX62$^-$, CD4$^+$, MHCII$^+$, and CD86$^+$ cells, corresponding to pDCs in rats (23). Twenty-four hours after DNA administration, we observed a 100 and 130% increase in the pDC number in P- compared to saline- and Met-P-injected tumors, respectively (Fig. 4A). Similarly, the number of tumor-infiltrating NK cells was increase by 190 and 100%, 24 h after native plasmid compared to saline and methylated plasmid injection, respectively (Fig. 4B). These last results were confirmed 24 h after DNA injection by immunohistologic analysis of the tumors. As shown in Fig. 4C, only a few NK cells were present within the saline- or methylated plasmid-treated tumors, whereas tumors injected with native plasmid were heavily infiltrated by this cell type. As IL-18 has been described to attract pDCs (24) which are known to produce chemokines inducing NK cell migration (25), we can thus speculate that plasmidic CpG motifs induce IL-18 expression, leading to pDC recruitment which in turn attracts NK cells.

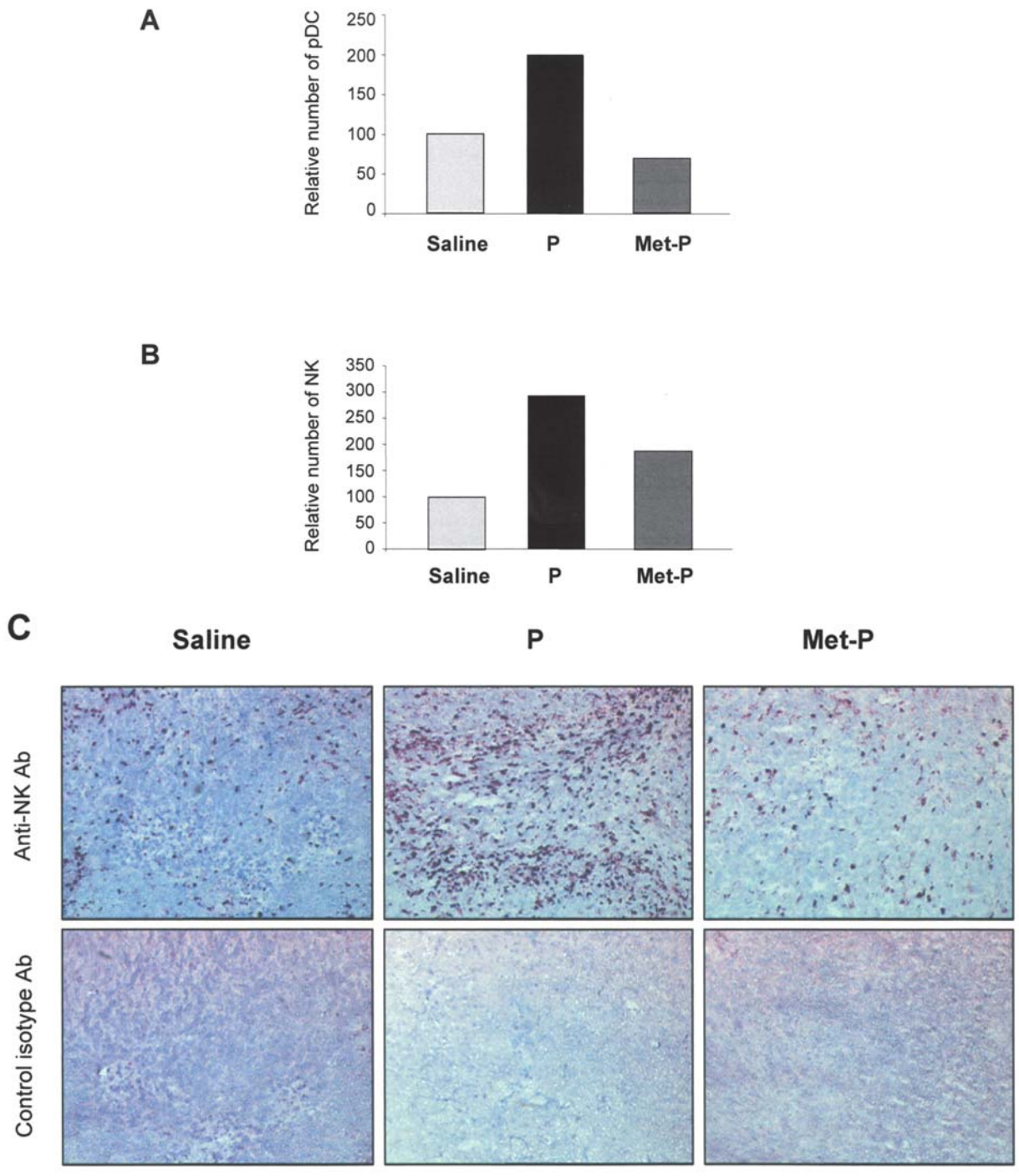

Figure 4. Analysis of tumor-infiltrating plasmacytoid dendritic cells (pDC) and natural killer (NK) cells after plasmidic CpG injection. Twenty-four hours after saline, P or Met-P intratumoral injection, the experimental liver metastases were pooled and dissociated, and a population enriched in tumor-infiltrating immune cells was isolated by selection of low-density cells. (A) The relative number of tumor-infiltrating plasmacytoid dendritic cells. $OX62^-$ low-density cells were isolated using anti-CD4-coated magnetic beads. The selected population was labeled with anti-CD4, -MHC II and -CD86 antibodies and analyzed by flow cytometry. The relative number of pDCs in each experimental condition corresponded to the number of $CD4^+$, MHC II^+, and $CD86^+$ cells normalized to the same number of total cells within the tumors. The results obtained in the saline condition were assigned the value of 100% (arbitrary units). Data are representative of three independent experiments. (B) The relative number of tumor-infiltrating NK cells. $OX62^-$ low-density cells were enriched in NK cells using anti-NK-coated magnetic beads. The selected population was then labeled with anti-NK, -MHC II and -γδ antibodies and analyzed by flow cytometry. The relative number of NK cells in each experimental condition corresponded to the number of NK^+, MHC II^-, and $\gamma\delta^-$ cells normalized to the same number of total cells within the tumors. The results obtained in the saline condition were assigned the value of 100% (arbitrary units). Data are representative of three independent experiments. (C) Immunohistologic analysis of tumor cryosections 24 h after saline, P or Met-P intratumoral injection. Upper panels, immunolabeling of NK cells. Lower panels, immunolabeling of corresponding sections with secondary antibodies alone. Original magnification for all panels, x10.

In addition to cancer cell destruction allowing tumor antigen release, an optimal antitumor immune response requires recruitment and activation of immune cells, creation of a proinflammatory environment and the presence of danger signals (26,27). Taken together, our data suggest that bacterial CpG motifs present in plasmid DNA are involved in

the generation of a tumor microenvironment facilitating the triggering of an antitumoral immune response.

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