Comparative Proteomics Study Reveals That Bacterial CpG Motifs Induce Tumor Cell Autophagy in Vitro and in Vivo*

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Unmethylated CpG dinucleotides, present in bacterial DNA, are recognized in vertebrates via the Toll-like receptor 9 (TLR9) and are known to act as an anticancer agent by stimulating immune cells to induce a proinflammatory response. Although the effects of CpG-oligodeoxynucleotides (CpG-ODNs) in immune cells have been widely studied, little is known regarding their molecular effects in TLR9-positive tumor cells. To better understand the role of these bacterial motifs in cancer cells, we analyzed proteome modifications induced in TLR9-positive tumor cells in vitro and in vivo after CpG-ODN treatment in a rat colon carcinoma model. Proteomics analysis of tumor cells by two-dimensional gel electrophoresis followed by mass spectrometry identified several proteins modulated by bacterial CpG motifs. Among them, several are related to autophagy including potential autophagic substrates. In addition, we observed an increased glyceraldehyde-3-phosphate dehydrogenase expression, which has been shown to be sufficient to trigger an autophagic process. Autophagy is a self-digestion pathway whereby cytoplasmic material is sequestered by a structure termed the autophagosome for subsequent degradation and recycling. As bacteria are known to trigger autophagy, we assessed whether bacterial CpG motifs might induce autophagy in TLR9-positive tumor cells. We showed that CpG-ODN can induce autophagy in rodent and human tumor cell lines and was TLR9-dependent. In addition, an increase in the number of autophagosomes can also be observed in vivo after CpG motif intratumoral injection. Our findings bring new insights on the effect of bacterial CpG motifs in tumor cells and may be relevant for cancer treatment and more generally for gene therapy approaches in TLR9-positive tissues.

The CpG dinucleotides are 4-fold more frequent in bacterial than in mammalian DNA, and they are generally methylated in mammals and unmethylated in bacteria (1). These differences lead the immune system to recognize unmethylated CpG sequences as a “danger signal” indicating a potential pathogen infection (2). In immune cells, recognition of these pathogen-associated molecular patterns (PAMPs) is mediated by the Toll-like receptor 9 (TLR9) (3) and followed by downstream signaling events leading to the activation of target genes (4). As a consequence, bacterial CpG motifs trigger pleiotropic effects in immune cells such as proliferation, activation, and cytokine/chemokine secretion (1). These proinflammatory properties led to the use of oligodeoxynucleotides (ODNs) bearing CpG motifs (CpG-ODNs) in preclinical and clinical studies to induce immune-mediated antitumoral effects in various kinds of cancers (5, 6). Although previously considered to be restricted to immune cells, expression of TLR9 by non-immune cells is now largely documented. It has recently been demonstrated that primary epithelial cells constituting a barrier between the organism and the external milieu express different TLRs (7–10). In addition, various kinds of tumor cells, including colon cancer cells, also express TLRs (11–13), and TLR9 agonists have been proposed to be involved in growth and survival of human myeloma cells (14, 15) as well as in invasion of human astrocytoma, glioblastoma, and breast cancer cells in vitro (16). Inversely CpG-ODNs have been shown to have antiproliferative and proapoptotic effects in vitro in human lung (17), breast (18), prostate (19), and colon (20) cancer cells as well as in murine glioma cells (21). Hence

1 The abbreviations used are: PAMP, pathogen-associated molecular pattern; ANXA1, annexin A1; CMA, chaperone-mediated autophagy; ODN, oligodeoxynucleotide; 2-D, two-dimensional; GFP, green fluorescent protein; GRP78, 78-kDa glucose-regulated protein; HEK, human embryonic kidney; LC3, microtubule-associated protein 1 light chain 3 protein; Met-P, SssI-methylated plasmid; P, native plasmid; PGAM1, phosphoglycerate mutase 1; PRoB, DHD/K12/PRoB; RH, rehydration buffer; TLR, Toll-like receptor; NCBI nr, National Center for Biotechnology non-redundant.
CpG-induced Autophagy in Cancer Cells

CpG effects in non-immune cells, and particularly in tumor cells, are still controversial, and this is, however, a crucial point as various cancer gene therapy clinical trials involve plasmid DNA or CpG-ODNs. In the present work, we analyzed proteome modifications induced in TLR9-positive colon cancer cells in vitro and in vivo 24 h after CpG-ODN treatment. As some of the modulated proteins were potentially related to autophagy, a process in which cellular organelles and bulk cytoplasm are targeted for degradation in lysosomes (22), we next analyzed the ability of bacterial CpG motifs to induce tumor cell autophagy. Our results show that, in addition to their known immunostimulatory properties, bacterial CpG motifs could exert a direct effect on TLR9-positive tumor cells in vitro and in vivo through the induction of autophagy.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Plasmids—The following sequences of the phosphorothioate-modified ODNs (Eurogentec, Seraing, Belgium) were used: for experiments in rodent cells (or human embryonal kidney (HEK) 293 cells expressing the murine TLR9): CpG-ODN 1826, 5’-TCCATGACTTCTCAGCTTT-3’; and control ODN, 5’-TCCATGACTTCTCAGCTTT-3’; for experiments in human cells: CpG-ODN 2006, 5’-TCTGACTTCTCAGCTTT-3’; and control ODN, 5’-TCTGACTTCTCAGCTTT-3’. The pcDNA3-GFP plasmid contains the cytosine deaminase gene under the control of the cytomegalovirus promoter (23). The pLC3-GFP plasmid expresses the LC3-GFP fusion protein (24).

Plasmid Methylation—Plasmid DNA was prepared using the Qiagen EndoFree Plasmid Mega kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions (endotoxins <0.1 enzyme unit/μg of DNA). The plasmid (200 μg in a 1-ml final volume) was methylated with SssI CpG methylase (New England Biolabs, Ozyme, St. Quentin en Yvelines, France) as described previously (25).

Cell Culture—DHD/K12/PROb (PROb) rat colon cancer cells (26) and human breast cancer MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) at 37 °C and 8 and 5% CO2, respectively. Human prostate cancer PC-3 cells were maintained at 37 °C in 5% CO2 in RPMI 1640 medium supplemented with 5% fetal bovine serum. HEK 293 cells stably transfected with the murine TLR9 gene (293 TLR9) or with the empty plasmid (293 Mock) were purchased from InvivoGen (Toulouse, France). Cells were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 10 μg/ml blasticidin (InvivoGen). For proteome analysis, PROb cells were plated into 150-mm tissue culture dishes in the presence of CpG-ODN or control ODN (10 μg/ml) or rapamycin (100 nm, Merck Chemicals) or DMSO as a control for 24 h. For electron microscopy analysis, PROb cells were plated in 6-well plates in the presence of CpG-ODN or control ODN at a final concentration of 10 μg/ml for 40 h. Medium was removed, and the cells were then cultured in complete medium in the presence or in the absence of lysosomal protease inhibitors E64d (10 μg/ml; Sigma) and pepstatin A (10 μg/ml; Merck) for the last 8 h of incubation.

TLR9 RT-PCR Analysis—For tissue RNA isolation, PROb tumors and healthy colon were reduced into powder mechanically in liquid nitrogen. Total RNA from tissues and cell lines was prepared by TRIzol extraction according to the manufacturer’s instructions (Invitrogen). Genomic DNA was removed by DNA-free DNase (Ambion, Courtaboeuf, France), and cDNA was synthesized using 1 μg of total RNA in a total reaction volume of 20 μl using SuperScript III reverse transcriptase and random hexamer primers (Invitrogen) according to the manufacturer’s instructions. cDNAs (100 ng) were amplified with Taq Platinum (Invitrogen). The human and rat TLR9 sequences were obtained from GenBank™ (accession numbers AF245704 and AY191271, respectively), and the following primers used in this study were purchased from Prologix (Paris, France): hTLR9-forward, 5’-GGTACCCACTTCTCCATG-3’ (positions 301–318); hTLR9-reverse, 5’-GGGACAGCATGATGTTTGG-3’ (positions 340–360); rTLR9-forward, 5’-AGGCTTTTATAGCCTTTCCG-3’ (positions 1321–1340); and rTLR9-reverse, 5’-AGTCCGACTCTCAGTAAG-3’ (positions 1893–1912). After an initial denaturation step (94 °C for 5 min), PCRs were performed for 35 cycles. Each cycle consisted of denaturation (94 °C for 1 min), annealing (54 °C for 45 s), and an elongation step (72 °C for 1 min) followed by an additional extension step (72 °C for 7 min). PCR products were separated in a 1.5% agarose gel containing ethidium bromide and were photographed under UV light.

Transfection—PROb, MCF-7, PC-3, and HEK 293 cells were transfected using Nucleofector I (Amaxis Biosystems, Cologne, Germany) according to the manufacturer’s instructions using X-05, P-20, T-13, and Q-01 programs, respectively. Briefly, cells were resuspended in the electroporation V-buffer at a final concentration of 1.5 × 106 (PROb and HEK 293) or 2 × 107 (MCF-7 and PC-3) cells/ml and transfected with 2 μg of pLC3-GFP plasmid in the absence or in the presence of 1 μg of CpG-ODN or 1 μg of control ODN. After electroporation, cells were transferred in complete medium containing or not 3-methyladenine (10 μM; Sigma-Aldrich) and cultured for 24 h in 6-well plates. Cells were then trypsinized, placed on coverslips, and cultured for 24 h in complete medium or medium containing 20 nm rapamycin (Merck Chemicals).

Experimental Liver Metastasis Generation—For in vivo 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. Rats were anesthetized, the liver was surgically exposed, and 1.5 × 106 PROb tumor cells in 100 μl were injected under the Glisson capsule. Fifteen days later, the rats had surgery, the liver was exposed, and 100 μl of CpG-ODN 1826 or control ODN (0.1 mg/ml) or native plasmid (P) or SssI-methylated plasmid (Met-P) DNA solution (1 mg/ml) were directly injected into the tumor. Twelve hours after injection, the animals were sacrificed, and the tumors were removed for analysis.

Sample Preparation for Proteome Analysis—For tumor proteome analysis, PROb tumors were reduced into powder mechanically in liquid nitrogen, and powdered tumors were resuspended in ultrapure water supplemented with aprotinin (2 μg/ml), pepstatin (1 μM), leupeptin (1 μg/ml), and iodoacetamide (1 mM). The suspension was sonicated (4 × 15 s, 4 °C, 40 watts) and then supplemented with urea, thiourea, CHAPS, and Triton X-100 to get a final concentration of 7 M, 2 M, 4%, and 0.24%, respectively (rehydration (RH) buffer). Protein solubilization was achieved by sonication (3 × 7 min at room temperature, 200 watts; Deltasonic, Meaux, France) and a final incubation of 30 min at 15 °C. After centrifugation (22,000 × g, 30 min, 15 °C), the supernatant containing soluble proteins was deep frozen in liquid nitrogen and stored at −80 °C until use. For PROb cell proteome analysis, the cells were washed twice in sucrose buffer (250 mM sucrose, 10 mM Heps, pH 7.4) and scraped in RH buffer. The resulting cell lysates were then incubated in the presence of spermidine at a final concentration of 10 mM for 30 min at 15 °C. After centrifugation (12,000 × g, 30 min, 15 °C), the supernatants were collected for protein concentration determination with the Bradford assay (Bio-Rad) using bovine serum albumin in RH buffer as standard curve.

Two-dimensional (2-D) Gel Electrophoresis and Protein Staining—2-D gel electrophoresis (pH range 3–10) and protein staining were performed as described previously (27). Gels were scanned using a
After a 20–24-h incubation at 37 °C, the incubation volume was adjusted to 100 μl with ultrapure water and acidified with 25 μl of formic acid at 25% (5% final concentration). Tryptic peptides were extruded from the gel by adding 125 μl of acetonitrile. After a 15-min incubation at room temperature, the gel pieces were spun down (8000 × g, 5 min, room temperature), the supernatant was collected, and the pellet was overlaid with 50 μl of acetonitrile and vortexed to complete peptide extraction. The extracted material was pooled with the supernatant and vacuum-dried. Lysates were solubilized in 10 μl of 5% formic acid, 20% methanol and stored at −20 °C until used. All samples were analyzed by micro-LC/ESI/MS/MS on an LTQ/FT-Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA) coupled with pumps and autosampler under standard conditions: capillary temperature, 275 °C; and source voltage, 4500 V. Helium was used as the collision gas. Experiments were done in parallel mode (survey at 60,000 resolution and five data-dependent ion trap MS/MS scans per cycle) (Top 5). The MS/MS parameters were as follows: isolation width, 3; collision energy, 35%; micro-HPLC Surveyor system (Thermo Fisher); 60-min gradient; and BioBasic C18 (Thermo Scientific) column (100 × 0.32 mm). Such high mass accuracy on the precursor ion allowed the elimination of virtually any false positive peptide identifications, suggesting that peptides that do not match the specificity of the protease used in the digestion should not automatically be considered as false positives. Acquired MS/MS spectra were interpreted using Mascot version 2.2.0 (Matrix Science, London, UK) in-house software. Search parameters were set as follows: enzyme specificity, trypsin; one missed cleavage permitted; fixed modification, carbamidomethylation of cysteine; variable modification, methionine oxidation; mass tolerance for precursor ions, 0.3 Da; significance threshold, p < 0.05; and expect value threshold, 0.001 (to select ions with the highest individual score). Both b and y ion series were used to search against UniProt KB/UniProtKB/Trembl (database version 51.6; 257,964 sequence entries) or NCBI nr (February 16, 2007; 4,626,804 sequence entries) databases. A species restriction to mammals was applied because the only expected contaminations could be from human (mainly keratins) or bovine (fetal calf serum proteins in cell culture medium) origins (UniProt KB/Swiss-Prot/TrEMBL, 49,887 sequence entries; NCBI nr, 551,056 sequence entries). In case of peptides matching to multiple members of a protein family, the presented protein was selected based on both the highest score and the highest number of matching peptides.

Fluorescence Confocal Microscopy—Forty-eight hours after transfection, cells were fixed with PBS containing 1% formaldehyde for 20 min at room temperature, and coverslips were mounted in Mowiol mounting medium. Slides were examined by confocal microscopy using the Zeiss 510 Meta laser scanning microscope. Cells were scored without prior knowledge of the experimental conditions.

RESULTS

CpG-ODN-induced Effects on TLR9-positive Colon Cancer Cells in Vitro and in Vivo—To investigate the molecular events underlying the various effects of CpG-ODN reported in tumor cells (14–21), we analyzed proteome modifications induced in the PROb TLR9-positive (supplemental Fig. S1A) rat colon carcinoma cell line after CpG-ODN treatment. As a similar uptake of cyanine-3-labeled CpG-ODN can be observed in PROb cells after transfection or addition within the culture medium (supplemental Fig. S2A), the cells were incubated for 24 h with CpG-ODN or control ODN, and soluble proteins were then separated by 2-D gel electrophoresis. For each condition, triplicate experiments were independently performed. For the analysis of in vivo effects, experimental liver metastases from colon carcinoma were generated by injection of PROb cells under the liver capsule of syngenic rats. Twenty-four hours after CpG-ODN or control ODN injection, tumors were removed, and soluble proteins were separated by 2-D gel electrophoresis. Four independent tumors were analyzed in each condition. Fig. 1 shows representative 2-D gel sections of tumor cells or tumors treated with CpG-ODN or control ODN. After gel-to-gel matching and normalization, protein spots displaying differential expression patterns among the CpG-ODN and control ODN-treated samples were digested and identified by LC/ESI/MS/MS. The proteins modulated in tumor cells upon CpG-ODN action in vitro and in vivo are presented in Tables I and II, respectively.

Biological Pathways Affected by CpG-ODN Treatment—We observed a majority of down-regulated proteins both in vitro and in vivo. In vitro, one overexpressed and six underexpressed proteins were reproducibly detected from CpG-ODN-treated cells compared with control ODN-treated cells. The identified proteins were grouped in the following different functional classes: 1) metabolism, 2) chaperoning and stress-related, and 3) miscellaneous. In vivo, one overexpressed and 10 underexpressed proteins were detected from CpG-ODN-injected tumors compared with control ODN-injected tumors. In addition to the functional classes found in vitro, three
proteins related to translation and protein synthesis were found to be modulated. With the exception of GRP78 (28), none of the *in vitro* or *in vivo* modulated proteins have been reported previously to be regulated by bacterial CpG motifs.

We first focused our attention on the two proteins that were found to be down-regulated by CpG-ODN both *in vitro* and *in vivo* (annexin A1 (ANXA1) (Fig. 2A) and phosphoglycerate mutase 1 (PGAM1) (Fig. 2B)). ANXA1, which is usually up-regulated in colon cancer (29), is an endogenous anti-inflammatory protein involved in many cellular functions, such as membrane aggregation, inflammation, phagocytosis, proliferation, and apoptosis (30). PGAM1 is a glycolytic enzyme interconverting 2- and 3-phosphoglycerates. Both ANXA1 and PGAM1 are known to be degraded by chaperone-medi-
down-regulated proteins, pyruvate carboxylase (Fig. 2), and citrate synthase (Fig. 2D), which are involved in gluconeogenesis and tricarboxylic acid cycle, respectively, have been described to be degraded during autophagy (33, 34). Finally an increased expression of glyceraldehyde-3-phosphate dehydrogenase, the only significantly up-regulated protein after CpG in vitro treatment (Fig. 2E), was recently identified as sufficient by itself to stimulate autophagy (35, 36). Autophagy is a self-digestion pathway involved in protein and organelle degradation for recycling (24), whereas material degradation occurs (24), which is a prosurvival mechanism, excessive autophagy can cause cell death (37). As the different prosurvival or proapoptotic effects of CpG-ODN in tumor cells could be explained by an upstream autophagy induction, these data prompted us to assess whether bacterial CpG motifs might induce autophagy in TLR9-positive tumor cells.

Assessment of CpG-ODN-induced Autophagy in TLR9-positive Cancer Cell Lines in Vitro—To test the hypothesis of CpG-mediated autophagy induction in tumor cells, we transfected PROb cells with control or CpG-ODN and the pLC3-GFP plasmid. This plasmid encodes for the microtubule-associated protein 1 light chain 3 protein (LC3), which is a human homologue of the yeast ATG8, an essential autophagy protein. Autophagosome formation can be scored by immunofluorescence microscopy as a transition of LC3 from its diffuse cytosolic appearance to a membrane-associated, punctate intracellular distribution (24). Fig. 3A displays representative photographs of PROb cells transfected with the pLC3-GFP plasmid and control or CpG-ODN. As shown in Fig. 3B, the presence of CpG-ODN induced a significant increase in the number of autophagic cells compared with transfection in the presence of control ODN and with pLC3-GFP transfection alone. This increase was similar to the induction observed in the presence of the autophagy inducer rapamycin (38) and can be reverted in the presence of the autophagy inhibitor 3-methyladenine (39). A similar increase in the number of PROb autophagic cells was observed when CpG-ODN was added in the culture medium (supplemental Fig. S2B). As autophagosomes ultimately fuse with lysosomes to generate single membraned structures termed autolysosomes where material degradation occurs (24), we then mon-

### Table I

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<th>Spot no.</th>
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*Numbers refer to the relative levels of each protein from CpG-ODN-injected tumors compared with those from control ODN-injected tumors. 

**Numbers refer to the relative levels of each protein from CpG-ODN-treated cells compared with those from control ODN-treated cells.

### Table II

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*Numbers refer to the relative levels of each protein from CpG-ODN-injected tumors compared with those from control ODN-injected tumors. 

**NCBI nr database (all others accession numbers are from Swiss-Prot/TrEMBL database).
monitored the number of autophagic vesicles (autophagosomes and autolysosomes) by transmission electron microscopy after incubation with control or CpG-ODN. Fig. 3 displays representative micrographs of PROb cells cultured in the presence of control or CpG-ODN. We observed a significant increase in the number of autophagosomes and autolysosomes in the presence of CpG-ODN compared with control ODN (Fig. 3D). To analyze whether this accumulation of autophagosomes was due to an increased formation or a decreased degradation, this experiment was also performed in the presence of lysosomal protease inhibitors (E64d and pepstatin A) (Fig. 3E). The accumulation of autolysosomes in the presence of inhibitors was further increased in the presence of CpG-ODN, indicating an induction, rather than a blockade, of an autophagic process by the CpG-ODN (Fig. 3F).

Finally to determine whether CpG-induced autophagy was a more general process in TLR9-positive (supplemental Fig. S1B) cancer cell lines, we transfected the MCF-7 human breast tumor cell line and the PC-3 human prostate carcinoma cells in vitro with control or CpG-ODN and the pLC3-GFP plasmid. Fig. 4, A and B, displays representative photographs of MCF-7 and PC-3 cells transfected with the pLC3-GFP plasmid and control or CpG-ODN. As shown in Fig. 4, C and D, the presence of CpG-ODN induced a significant increase in the number of autophagic cells compared with control conditions in both cell lines.

Requirement of TLR9 for CpG-induced Autophagy—To determine whether TLR9 was required for CpG-induced autophagy, we then used HEK 293 cells stably expressing (293 TLR9) or not (293 Mock) the murine TLR9. Both kinds of cells were transfected with the pLC3-GFP plasmid and CpG-ODN.
or control ODN. Representative photographs of autophagic or control 293 TLR9 cells are presented Fig. 5A. In the 293 TLR9 cell line, we observed a 6-fold induction of autophagy after CpG-ODN treatment compared with control conditions (Fig. 5B). Although rapamycin was able to induce autophagy in 293 Mock cells indicating that the autophagic pathway is func-

**Fig. 3.** Autophagy induction by CpG-ODN in the rat PROb colon carcinoma cell line. A, representative photographs of CpG-ODN and control ODN-transfected cells are presented. Scale bars, 10 μm. B, effect of CpG-ODN. The number of autophagic cells is expressed as the median with 95% confidence intervals (350 GFP-positive cells counted in each condition). Cells were scored without prior knowledge of the experimental conditions. Data are representative of three independent experiments. 3-MA, 3-methyladenine. C and E, transmission electron microscopy after incubation with control or CpG-ODN in the absence (C) or in the presence of lysosomal protease inhibitors (E). Arrows indicate autophagosomes, and arrowheads indicate autolysosomes. D and F, 50 cells per condition were randomly chosen, and the number of autophagosomes and autolysosomes were quantified without prior knowledge of the experimental conditions. Each condition was run in triplicate. Results are expressed as the median number of autophagosomes or autolysosomes per cell and per condition with 95% confidence intervals. NS, not significant; I, inhibitors.
tional, no difference was observed in these cells treated with CpG-ODN compared with control ODN, suggesting that the effect is TLR9-dependent (Fig. 5).

Assessment of CpG-ODN-induced Autophagy in TLR9-positive Tumor Cells in Vivo—To assess the relevance of our in vitro findings, we next examined whether CpG motifs could also induce autophagy in vivo in an experimental liver metastases model from colon carcinoma. We compared the number of autophagosomes in PROb tumors after intratumoral injection of CpG-ODN or control ODN using transmission electron microscopy (Fig. 6A). The median number of autophagosomes per field increased significantly in CpG-ODN-compared with control ODN-injected tumors (Fig. 6B). As bacterial plasmids, which present unmethylated CpG motifs, are widely used in preclinical and clinical cancer gene therapy, the same experiment was performed in tumors injected with a P, bearing active CpG sequences, or a Met-P in which the effect of CpG sequences has been abolished by in vitro methylation (25). Similarly the median number of autophagosomes per field increased significantly in P-compared with Met-P-injected tumors (Fig. 6, C and D). Thus, these data demonstrate for the first time that the presence of bacterial CpG motifs is associated with an increase of autophagy within the tumors in vivo.

Fig. 4. Autophagy induction by CpG-ODN in the human MCF-7 and PC-3 cancer cell lines. A and B, 48 h after transfection, cells were treated as described in Fig. 1A. Representative photographs of CpG-ODN- and control ODN-transfected cells in MCF-7 and PC-3 cell lines, respectively, are presented. Scale bars, 10 μm. C and D, the number of autophagic cells expressed as the median with 95% confidence intervals (700 GFP-positive cells counted in each condition) obtained with MCF-7 and PC-3 cells, respectively. Cells were scored without prior knowledge of the experimental conditions. Data are representative of three independent experiments. NS, not significant; 3-MA, 3-methyladenine.
DISCUSSION

Bacterial CpG motifs are PAMPs that are recognized by immune cells and trigger a proinflammatory immune response. Although the effects of CpG-ODN in immune cells have been widely studied, little is known regarding their molecular effects in tumor cells. In the present study, we demonstrated for the first time that bacterial CpG motifs can modulate tumor cell proteome in vitro and in vivo. Among the different proteins identified, several are related to autophagy. These proteins include potential autophagic substrates (ANXA1, PGAM1, pyruvate carboxylase, and citrate synthase) and glyceraldehyde-3-phosphate dehydrogenase for which

![Image of HEK 293 TLR9 cells with CpG-ODN and control ODN](image)

**Fig. 5.** TLR9 requirement for autophagy induction by CpG-ODN. A, 48 h after transfection, cells were treated as described in Fig. 1A. Representative photographs of CpG-ODN- and control ODN-transfected cells in the 293 TLR9 cell line are shown. Scale bars, 10 μm. The number of autophagic cells expressed as the median with 95% confidence intervals (125 GFP-positive cells counted in each condition) obtained with 293 TLR9 cells (B) and 293 Mock (C) is shown. Cells were scored without prior knowledge of the experimental conditions. Data are representative of three independent experiments. NS, not significant; 3-MA, 3-methyladenine.
an increased expression was shown to be sufficient to trigger an autophagic process (35, 36). These observations were further supported by the *in vivo* down-regulation of eukaryotic elongation factor 2, a key player involved in protein translation, as translation decreases toward an adaptative protein synthesis during autophagy (40). Interestingly eukaryotic elongation factor 2 was also found to be down-regulated in mouse small intestine after starvation, a process likely involving autophagy (41).

Autophagy is a process in which cellular organelles and bulk cytoplasm are targeted for degradation in lysosomes (22). Macroautophagy and CMA are two different types of autophagy that differ in the procedures used for delivery of substrates to lysosomes and the nature of these substrates. In macroautophagy (autophagy), cytoplasmic material is sequestered by a double membrane structure termed the autophagosome for subsequent degradation and recycling (22). Pyruvate carboxylase and citrate synthase were found to be degraded during autophagy (33, 34). In CMA, cytosolic proteins are delivered one by one into lysosomes (42), and ANXA1 and PGAM1 were described as substrates of CMA (31, 32). However, both autophagy and CMA are maximally activated by cellular stress, and a sequential switch from macroautophagy to CMA has been observed during starvation (43).

As macroautophagy is activated in response to extracellular pathogens such as bacteria (44), it was reasonable to think that bacterial CpG motifs, which are known as a danger signal, could trigger an autophagic process. In this study, we showed that CpG-ODN can induce autophagy in tumor cell lines from colon, breast, and prostate cancers and that TLR9 was required for this process. In addition, an increase in the number of autophagosomes can also be observed *in vivo* after intratumoral injection of CpG-ODN or plasmidic CpG motifs in a rat model of liver metastasis.

Depending on the conditions, autophagy is able to induce cell survival or cell death (37). Thus, our data could reconcile the apparently conflicting results showing prosurvival (14–16) or proapoptotic (17–21) effects of CpG-ODN in tumor cells. Finally autophagy is also considered as an innate immunity mechanism because of its role in the removal of intracellular pathogens (44). Recent works have demonstrated that different PAMPs are able to induce autophagy via TLR3, -4, and -7 in macrophages (45, 46). Bacterial CpG motifs are defined as...
a danger signal activating immune cells. In the present study, we demonstrated that these motifs can also be sensed as a danger signal in non-immune cells and trigger an autophagic process in TLR9-positive tumor cells, extending the link between TLRs and autophagy.

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