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Targeting CpG Oligonucleotides to the Lymph Node by Nanoparticles Elicits Efficient Antitumoral Immunity

Carole Bourquin,* David Anz,† Klaus Zwiorek,‡ Anna-Lisa Lanz,* Sebastian Fuchs,* Sarah Weigel,* Cornelia Wurzenberger,* Philip von der Borch,* Michaela Golic,* Stefan Moder,* Gerhard Winter,† Conrad Coester,† and Stefan Endres*

Viral nucleic acids are recognized by specific pattern-recognition receptors of the Toll-like and RIG-I-like receptor families. Synthetic DNA and RNA oligonucleotides can activate the immune system through these receptors and potentiate Ab and CD8 cytotoxic responses to Ags. Systemic application of immunostimulatory oligonucleotides however also results in a generalized, non-Ag-specific stimulation of the immune system. In this study, we have dissociated the induction of an Ag-specific response from the systemic immune activation generally associated with immunostimulatory oligonucleotides. Delivery of CpG oligodeoxynucleotides that bind TLR9 by cationized gelatin-based nanoparticles potentiates the in vivo generation of an Ag-specific cytotoxic T cell and Ab response. Furthermore, immunization with CpG-loaded nanoparticles induces a protective antitumoral response in a murine model of melanoma. The systemic release of proinflammatory cytokines and widespread immunostimulation associated with free CpG is however completely abolished. In addition, we show that gelatin nanoparticle formulation prevents the destruction of lymphoid follicles mediated by CpG. Nanoparticle-delivered CpG, in contrast to free CpG, are selectively targeted to APCs in the lymph nodes where they mediate local immune stimulation. We describe a novel strategy to target immunostimulatory oligonucleotides to the initiation site of the immune response while at the same time protecting from an indiscriminate and generalized activation of the immune system. The Journal of Immunology, 2008, 181: 2990–2998.

Viral infections induce strong innate immunity that is initiated by the recognition of viral components through specific receptors (1). In particular, nucleic acid sequences and structures that are specific to viruses are recognized by the pattern-recognition receptors TLR3 and melanoma differentiation Ag-5 (MDA5) (double-stranded RNA), TLR7 (single-stranded RNA sequences), TLR9 (CpG DNA sequences), and retinoic acid-inducible gene-1 (RIG-I) (5′-triphosphate RNA) (2–6). Exposure of APCs to these ligands induces rapid cellular activation followed by their maturation and migration to the lymph nodes (7). This activation process is critical for the induction of an adaptive, Ag-specific immune response associated with protective immunity. The strong immunostimulatory capacity of these ligands reflects an important therapeutic potential as vaccine adjuvants for the treatment of infections and tumors. For instance, we have recently shown that ssRNA oligonucleotides trigger an efficient Th1-type immune response to Ag with a strong CD8 component (8).

A major obstacle to the clinical application of RNA oligonucleotides as vaccine adjuvant is the need for an efficient delivery system in order for RNA to show immunostimulatory activity (3, 4, 8, 9). Furthermore, systemic application of viral nucleic acids leads to an unspecified, generalized activation of the immune system that may be deleterious (8, 10, 11). In this study, we examine the impact of nucleic acid delivery by gelatin nanoparticles (NP)7 on both the innate and adaptive immune responses in vivo. Gelatin-based nanoparticles present several advantages: gelatin is a natural macromolecule extracted from collagen that is biodegradable, nontoxic, and has been approved as a plasma expander for decades (12, 13). Gelatin nanoparticles present greater stability during storage than liposomal delivery systems, high stability in vivo after administration and ease of scale-up during manufacture (14). Highly homogeneous gelatin nanoparticles of a well-defined diameter can be generated by a two-step desolvation method (15).

To assess the effect of nucleic acid delivery by gelatin nanoparticles on immune responses, we selected CpG oligodeoxynucleotides (CpG), as this ligand for TLR9 represents the most potent known adjuvant for human CD8 T cell responses (16). We have recently described the uptake and activation of both human and murine APCs by CpG oligonucleotide-loaded gelatin nanoparticles (17). In this study, we demonstrate that gelatin nanoparticle delivery further enhances the CD8 T cell

4 Abbreviations used in this paper: NP, nanoparticle; DC, dendritic cell; Treg, regulatory T cell.

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response triggered by CpG oligonucleotides. In addition, immunization with nanoparticle-bound CpG results in a protective antitumor response that is Ag-specific. Remarkably, the unspecific activation of the innate immune system generally induced by free CpG oligonucleotides, characterized by the increase of proinflammatory cytokines in the serum, systemic activation of immune cells in secondary lymphoid organs and splenomegaly, is abolished. Gelatin nanoparticles selectively target CpG oligonucleotides to APCs in the lymph nodes where priming of the adaptive immune response takes place. We thus characterize a new formulation for immunostimulatory oligonucleotides that enhances their ability to induce a strong Th1-type immune

FIGURE 1. CpG-loaded nanoparticles potentiate a Th1-type immune response to OVA in vivo. C57BL/6 mice were immunized twice s.c. at a 14-day interval with OVA formulation together with 100 μg free CpG or the same amount of CpG complexed to NP (5% w/w). One week after the second immunization, spleen cells were isolated. A and B, The generation of OVA-specific CTL was assessed by flow cytometry using H-2k B-OVA257–264 peptide pentamers and an anti-CD8 mAb. C and D, Splenocytes were restimulated with the OVA257–264 peptide for 4 h and cytoplasmic expression of IFN-γ in CD8⁺ T cells was examined by flow cytometry. E, OVA-specific IgG, IgG1, and IgG2a were measured in serum samples by ELISA. A and C, Representative data from one experiment are gated on CD8⁺ T cells. Numbers indicate the percent of CD8⁺ cells that are OVA-pentamer⁺ or IFN-γ⁺. B, D, and E, Data show the mean values of individual mice (n = 5) ± SEM. Results are representative of two independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant.
response to Ag while at the same time protecting against a generalized activation of the immune system.

Materials and Methods

Mice

Female BALB/c and C57BL/6 mice were purchased from Harlan-Winkelmann. Mice were 5 to 12 wk of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

Reagents and cell lines

The phosphate-modified CpG oligodeoxynucleotide 1826 (5'-TCCATGACGTTCCTGACGTT-3') was obtained from Coley Pharmaceutical Group. For some experiments, Cy5-5'-tagged CpG (Metabion International) was used. Gelatin type A from porcine skin (175 Bloom), glutaraldehyde (25%), aceton, 1-ethyl-3-(3-dimethyl-amino propyl) carbodiimide hydrochloride (EDC), and (2-aminoethyl)-trimethylammonium chloride hydrochloride (chloramine chloride hydrochloride) were purchased from Sigma-Aldrich GmbH. For flow cytometry analysis, cells were stained with anti-mouse B220-PE, CD3-allophycocyanin, CD4-PE, CD8-PerCP, CD11b-PerCP, CD11c-allophycocyanin, CD69-FITC or PE, CD86-FITC, CD80-PE, MHC-II-PE and isotype controls (BD Biosciences). Chicken egg OVA was purchased from Sigma-Aldrich. The B16-F10 cell line and the OVA-transfected line B16-F10-OVA (B16-OVA) were a gift from Prof. T. Brocker (Institute for Immunology, Munich, Germany).

Preparation of cationized gelatin nanoparticles

Gelatin nanoparticles were prepared in the Division of Pharmaceutical Technology and Biopharmaceutics at the University of Munich as previously described (15). Subsequently, cationization of the nanoparticles was achieved through introduction of a quaternary amino group by covalent coupling of cholamine chloride hydrochloride onto the particle surface, as previously described for a gene delivery approach (17). Cationized particles prepared by this protocol were shown by Luminus amoebocyte lysate assay to be endotoxin free (14). The size of the prepared nanoparticles was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern). Average diameter was 272 nm (SD 33.3) with a polydispersity index under 0.1 and a potential of 14 mV. Particles were loaded with 5% (w/w) CpG or OVA as described (17). The nanoparticle dispersion was then entrapped and the supernatant was analyzed UV-spectrophotometrically at 260 nm wavelength for free CpG. Unloaded particles were stable in size and zeta potential when stored at 4 °C for several months. CpG-loaded particles were stable in size at 37 °C for at least 12 days. CpG release from the particles was slow with 3% release at pH 7.4 and 9% release at pH 5 after 12 days.

Induction of an OVA-specific immune response

In brief, 75 μg OVA complexed to nanoparticles (5% w/w) were used as Ag. They were injected s.c. together with 100 μg free CpG or 100 μg CpG loaded onto nanoparticles (5% w/w) on day 0 and day 14. Splenect and serum were collected on day 21. Freshly isolated splenocytes were incubated with ammonium chloride buffer for erythrocyte lysis and were stained with H-2kb-OVA257–264-PE pentamers (Proimmune) and anti-CD8-PerCP (BD Biosciences). For the detection of intracellular IFN-γ, cells were restimulated for 4 h. Cells were stained with CD8-PerCP, then fixed with 2% paraformaldehyde and treated with permeabilizing solution (0.5% BSA, 0.5% saponin, 0.02% sodium azide in PBS). The fixed cells were stained with FITC-conjugated anti-IFN-γ Ab for 25 min. The percentage of CD8+ T cells expressing IFN-γ was determined by flow cytometry. Serum Abs to OVA were determined by ELISA: 96-well plates were coated overnight with 10 μg/ml OVA in PBS and blocked 1 h with 1% BSA in PBS. After incubation of serum samples for 1 h at a dilution of 1/200, plates were washed with PBS/1% Tween 20. Goat anti-mouse IgG, IgG1, or IgG2a conjugated to HRP (Southern Biotechnologies Associates) was added at 1 μg/ml for 1 h. Plates were again washed and ELISA was developed by o-phenylenediamine (Sigma-Aldrich). Reaction was stopped by 1 M H2SO4 and OD was read by photometer at 450 nm.

Tumor challenge

Before tumor challenge, mice were immunized four times on days 0, 7, and 14 and at 2 mo with 50 μg OVA s.c. together with 100 μg free CpG or 100 μg CpG loaded onto nanoparticles (5% w/w). Three weeks after the last injection, 105 B16 or B16-OVA cells were injected subcutaneously in the right flank. Tumor growth was expressed as the product of the perpendicular diameters of individual tumors. Animals were euthanized when tumor size exceeded 225 mm2. The experiment was terminated when less than three mice per group remained.

In vivo uptake of CpG-nanoparticles and immunostimulation

For in vivo immunostimulation, 100 μg CpG alone or complexed to nanoparticles (CpG-NP; 5% w/w) were injected s.c. In some experiments, Cy5-tagged CpG was used. The draining and nondraining lymph nodes and spleen were isolated at the indicated time points. For analysis by flow cytometry, single cell suspensions were prepared, treated with ammonium chloride buffer to lyse erythrocytes, and stained with fluorochrome-coupled mAbs. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed using CellQuest software (BD Biosciences). Blood was obtained by retroorbital puncture; serum was prepared by centrifugation and stored at −20 °C.

Quantification of cytokines in serum and supernatant

Cytokine concentrations were determined by ELISA for IL-6 (BioSource) and IL-12p70 (BD Biosciences) according to the manufacturers’ protocol. IFN-α was measured according to the following protocol: rat mAb to mouse IFN-α (clone RMMA-1) was used as the capture Ab, rabbit polyclonal Ab to mouse IFN-α for detection (both from PBL Biomedical Laboratories) together with HRP-conjugated donkey Ab to rabbit IgG as the secondary reagent (Jackson ImmunoResearch Laboratories). Recombinant mouse IFN-α (PBL Biomedical Laboratories) was used as standard (IFN-α concentration in IU/ml).

Immunohistology and image acquisition

Cryostat sections of spleens were air-dried and fixed in ice-cold acetone for 10 min. Rat anti-mouse CD3, F4/80 (both from Abd Serotec), B220 and Ly6G (both from BD Biosciences) were used as primary Abs. Biotinylated donkey anti-rat and alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) served as secondary reagents. Detection of alkaline phosphatase was performed using new
FIGURE 3. Nanoparticle formulation abolishes in vivo splenocyte activation and systemic cytokine production by CpG. C57BL/6 mice were injected s.c. with 100 μg free CpG or NP-bound CpG. Control mice were injected with unloaded NP. A. Splenocytes were isolated on day 3 after injection and CD69 expression on lymphocyte populations was examined. Representative data from one experiment are gated on B cells (B220<sup>+</sup>, CD11c<sup>-</sup>), CD3<sup>+</sup> T cells, or NK cells, respectively. Numbers indicate the percentage of gated lymphocytes that are CD69<sup>+</sup>. B. Data show the mean of individual mice in one experiment (n = 5) ± SEM. *, without brackets: comparison to NP. C. Blood samples were taken 2, 6, and 22 h after injection and concentrations of IL-12p70, IL-6, and IFN-α were measured in serum. Data show the mean values of individual mice (control NP group for IL-12p70 and IFN-α: n = 1, all other groups: n = 5–6) ± SEM. Comparison to NP-CpG: *, p < 0.05; **, p < 0.01. Results are representative of two independent experiments.
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FIGURE 4. Nanoparticle formulation protects from lymphoid follicle destruction by CpG. A, C57BL/6 mice were injected s.c. three times at 3-day intervals with 100 µg free CpG or NP-bound CpG. Three days after the last injection, liver and spleen were stained for B220, F4/80, CD3, or Ly6G. B, C57BL/6 mice were injected twice as in A. Three days after the last injection, spleen weight was determined. Values for individual mice (dots) and mean value for each group (bar) are shown. C, The number of infiltrating cells per field (20- and 40-fold magnification for B220 and F4/80, respectively) were counted in liver sections shown in A. The mean of ten counted fields is shown. Results are representative of two independent experiments.

Measurement of IDO enzymatic activity
Lungs were isolated from mice 4 days after s.c. injection of 100 µg free CpG or 100 µg CpG loaded onto nanoparticles (5% w/w). Tissue homogenates were mixed with lysis buffer (BioRad Laboratories) and centrifuged. Supernatants were incubated with 100 mM tryptophane as substrate for 2 h. Protein concentration in lung homogenate supernatants was determined. IDO activity was expressed as ng kynurenine per mg protein per 2 h. Protein concentration in lung homogenate supernatants was determined using a modified detergent-compatible Bradford assay (BioRad Laboratories).

Regulatory T (Treg) cell suppression assay
CD4^+CD25^- effector cells, CD4^+CD25^- Treg cells and CD11c^- splenic dendritic cells (DC) were sorted using Magnetic Cell Isolation Kits (Miltenyi Biotech) according to the manufacturer’s instructions. The purity of Treg cells was greater than 97%. CD4^+CD25^- effector T cells (7.5 × 10^4 cells per well) were cultured in the presence of soluble anti-CD3 Ab (clone 145–2C11, 1 µg/ml; BD Biosciences) with 5 or 2.5 × 10^4 CD4^+CD25^- Treg cells and 3 × 10^5 CD11c^- splenic DCs. Cell proliferation was measured by BrdU incorporation (7.5 µM) for the last 18 h of a 3-day culture. BrdU incorporation was assessed by ELISA according to the manufacturer’s instructions (Roche) and chemiluminescence was measured in relative light units with a multilabel plate reader (Mithras, Berthold Technologies). All conditions were assayed in triplicate. Treg cell-mediated suppression was expressed as percentage of effector T cell proliferation without Treg cells. Negative suppression values were set to zero.

Statistics
All data are presented as mean ± SEM and were analyzed as appropriate by unpaired Student’s t test or by unpaired, one-way ANOVA with the Student-Newman-Keuls multiple-comparison test. Mean tumor size curves were plotted until two mice died or were euthanized. The last measured value was included in calculation of the mean for remaining time points. Comparisons in tumor size among groups were made using the Mann-Whitney U test for various time points. Comparisons among groups regarding survival time were made using the log-rank test. Significance was set at a p value of less than 0.05. Statistical analysis was performed using SPSS software.

Results
Nanoparticle-delivered CpG efficiently trigger an Ag-specific Th1-type immune response
To assess the immunostimulatory potential of nanoparticle-delivered CpG in vivo, mice were immunized twice at a 14-day interval with the model Ag OVA together with either CpG-loaded nanoparticles or free CpG. OVA was loaded onto gelatin nanoparticles before s.c. injection, as this Ag formulation induces an efficient immune response when combined with adjuvant (data not shown). In mice immunized with CpG-loaded nanoparticles, nearly 5% of splenic CD8^- T cells were specific for the MHC-I-restricted peptide OVA257–264 compared with 1.1% in mice treated without CpG (Fig. 1, A and B). A similar increase was seen for OVA-specific CD8^- T cells in peripheral blood (data not shown). In mice immunized with free CpG, specific CD8^- T cells levels were intermediate at 2.4%. To measure T cell function, splenocytes from immunized mice were restimulated with OVA257–264 and assessed for IFN-γ production. CpG-loaded nanoparticles clearly enhanced IFN-γ production in CD8^- T cells (Fig. 1, C and D). Furthermore, CpG-loaded...
nanoparticles potentiated the development of an OVA-specific IgG response as efficiently as free CpG (Fig. 1E). In particular, OVA-specific Abs of the IgG2a isotype, indicative of a Th1-type response, were enhanced. In contrast, OVA-specific IgG1 was similarly increased in all groups treated with OVA. In mice immunized with OVA together with the nanoparticle-bound control oligonucleotide 1982 that lacks CpG motifs, no increase in OVA-specific T cells was seen compared with mice immunized with OVA alone (data not shown). We thus demonstrate that nanoparticle-delivered CpG efficiently enhances CD8 T and B cell responses to Ag in vivo.

Nanoparticle delivery of CpG protects from generalized and unspecific immune activation

Free CpG not only functions as adjuvant to increase immune responses upon immunization with Ag but also induces generalized, non-Ag-specific immune activation in vivo, characterized by lymphocyte activation in the spleen and systemic release of inflammatory cytokines (11). To examine the unspecific immunostimulatory effect of CpG in nanoparticle formulation, mice were injected s.c. with 100 μg free CpG or the same amount of CpG complexed to nanoparticles. Expression of the early activation marker CD69 was assessed on splenic lymphocytes 18 h after injection. Remarkably, while free CpG strongly increased CD69 expression on spleen T cells, B cells and NK cells, the same amount of CpG bound to nanoparticles only marginally increased CD69 expression on all lymphocyte populations examined (Fig. 3, A and B). Indeed, the increase in CD69 expression was significant only for the B cell population but not for T cells or NK cells.

To further characterize the effect of nanoparticle formulation on CpG-induced immune activation, cytokine levels were measured in the serum of mice injected with 100 μg free CpG or the same amount of CpG complexed to nanoparticles. As expected, free CpG strongly increased serum levels of the proinflammatory cytokines IL12p40, IL12p70, IL-6, and IFN-α and the proinflammatory chemokine RANTES/CCL5 2 to 6 h post injection (Fig. 3C and data not shown). Strikingly, no increase in serum cytokine and chemokine levels was observed in mice injected with nanoparticle-bound CpG up to 22 h postinjection.

FIGURE 5. Nanoparticles target CpG to the draining lymph nodes. One hundred micrograms of Cy5-labeled free or NP-bound CpG was injected into the forelimb of C57BL/6 mice. Spleen, ipsilateral (draining), and contralateral lymph nodes were isolated at the indicated time points. A, Percentage of CpG-positive cells was determined by flow cytometry. Data show the mean values of individual mice (n = 2). B, Expression of the activation markers CD69, CD80, and MHC class II was examined for CpG-positive and CpG-negative conventional DCs (CD11b+/CD11c−) DC in ipsilateral lymph nodes on day 2 after injection. Control mice were injected with NP. Data show the mean values of individual mice (n = 5) ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant; compared with NP. ***, with brackets: comparison CpG− vs CpG+. C, Lymph node sizes were determined two-dimensionally by digital analysis of scaled photographs. Data show the mean values of individual mice (n = 3) ± SEM. ***, comparison to day 0. All results are representative of two independent experiments.

Nanoparticle delivery of CpG elicits a protective antitumor response

The development of a strong Th1-type response with a functional CD8 T cell component is a requirement for obtaining efficient antitumor immunity. To assess whether the Ag-specific response induced by CpG-loaded nanoparticles protected from the development of a tumor, immunized mice were challenged s.c. with either OVA-expressing B16 melanoma cells or with wild-type B16 cells. In mice immunized with OVA and nanoparticle-bound CpG, growth of the B16-OVA tumor was reduced compared with untreated mice or to mice immunized with OVA only (Fig. 2A). Indeed, tumor size was comparable to that of tumors in mice immunized with OVA and free CpG. The reduction in tumor growth was associated with an increase in survival (Fig. 2B). In contrast, development of wild-type B16 tumors was not reduced by immunization, indicating that antitumoral protection was Ag-specific.
Nanoparticle formulation prevents lymphoid follicle destruction by CpG

Repeated administration of free CpG in mice results in splenomegaly, disruption of splenic microarchitecture with destruction of lymphoid follicles and disappearance of germinal center B cells (18). Furthermore, lymphocytic infiltrates of the liver, hyperplasia of Kupffer cells, and hepatocellular degeneration are observed. We assessed the effect of nanoparticle formulation of CpG on splenic microarchitecture and hepatic infiltrates. Mice were injected s.c. two to three times with 100 μg free or nanoparticle-bound CpG and morphology of spleen and liver was assessed on day 12. Immunohistochemical staining of B cells (B220), T cells (CD3), granulocytes (Ly6G) and macrophages (F4/80) revealed a disruption of splenic follicular structure and a 3-fold increase in spleen weight following treatment with free CpG (Fig. 4, A and B). In addition, lymphocytic and myeloid infiltrations of the liver were observed (Fig. 4, A and C). In contrast, only moderate alterations of splenic microarchitecture and weight were detected in mice treated with CpG-loaded nanoparticles. No liver infiltrates were observed in these mice.

Nanoparticle formulation targets CpG to the draining lymph nodes

The striking differences in systemic immune activation between free and nanoparticle-bound CpG suggested that nanoparticle formulation could modify the in vivo distribution of CpG. To assess distribution and uptake of nanoparticle-bound CpG, nanoparticles loaded with Cy5-labeled CpG were injected subcutaneously. At 1, 3 and 7 days later, CpG uptake was measured in spleen and lymph nodes by flow cytometry. Strikingly, in mice injected with CpG-loaded nanoparticles, less than 1% of splenocytes were positive for CpG at any time point. In contrast, CpG was observed in nearly 8% of splenocytes in mice injected with free CpG (Fig. 5A). Nanoparticle-delivered CpG was however readily detected in the draining lymph nodes. In this study, ~25% of lymph node cells stained positive for CpG on day 1 in mice injected either with nanoparticle-bound or free CpG (Fig. 5A). A peak in CpG-positive cells was seen in both groups at day 3 with up to 50% positive cells. On day 7, CpG was still detectable in both treatment groups. We thus demonstrate that nanoparticle formulation selectively targets CpG to the draining lymph nodes and prevents transport of CpG to distant secondary lymphoid organs.

Uptake of nanoparticle-bound CpG in the draining lymph nodes was highest in APCs, with over 30% of DCs and 20% of B cells staining positive for CpG on day 2 after injection. Twelve percent of T cells were also positive for CpG. Uptake of CpG by DCs was associated with increased surface expression of CD69, CD80, and MHC II, indicating selective activation of CpG-positive DCs (Fig. 5B). Expression of the early activation marker CD69 was also increased, to a lesser extent, on CpG-negative DCs, suggesting bystander activation. In addition, as with free CpG, draining lymph nodes increased in size following s.c. injection of CpG-loaded nanoparticles (Fig. 5C). This change was detectable as early as 1 day after injection and increased on days 3 and 7. In contrast, no change in the size of contralateral lymph nodes was detected (data not shown). Thus, while systemic immunostimulation is prevented by formulation of CpG with gelatin nanoparticles, immune activation is preserved in the lymph nodes directly draining the injection site. Uptake of nanoparticle-delivered CpG in the draining lymph nodes leads to direct activation of APCs and to increased expression of MHC and costimulatory molecules.

**FIGURE 6.** Nanoparticle-bound CpG inhibits immunosuppressive mechanisms. A, C57BL/6 mice were injected s.c. with 100 μg free CpG or NP-bound CpG. Lungs were isolated on day 4 after injection and IDO enzymatic activity in tissue homogenates was examined. Data show the mean of individual mice (n = 3–6) ± SEM. *, without brackets: comparison to control group without CpG. B, CD4+/CD25+ effector T cells, CD4+/CD25+ Treg cells and CD11c+ splenic DCs from untreated BALB/c mice were coincubated at the indicated ratios. Suppression is expressed as percentage of effector T cell proliferation without Treg cells. p values were calculated only for the higher Treg cell: effector ratio. ***, without brackets: comparison to control without CpG, *, p < 0.05; **, p < 0.01.

Nanoparticle-bound CpG prevents activation of immunosuppressive mechanisms

Treatment with free CpG can up-regulate the rate-limiting tryptophan-catabolizing enzyme IDO within different organs (19, 20). DC-expressing IDO contribute to the induction of immune tolerance by inducing regulatory T cells (Treg cells) (21, 22). To investigate the effect of nanoparticle-bound CpG on systemic IDO activity, we measured IDO enzymatic activity in the lung after s.c. administration of free or particle-bound CpG. In contrast to free CpG that induced high IDO activity, no increase in IDO activity was observed following injection of particle-bound CpG (Fig. 6A). Furthermore, as previously described for free CpG (23), nanoparticle-bound CpG overcame suppression of effector T cell proliferation mediated by Treg cells (Fig. 6B). Thus, nanoparticle-bound CpG prevents or inhibits two major immunosuppressive mechanisms.

**Discussion**

The remarkable ability of CpG oligonucleotides to induce exceptionally strong and rapid CD8 T cell responses in patients marks CpG as an extremely promising vaccine adjuvant (16). In this study, we show that formulation of CpG with cationized gelatin nanoparticles still further enhances the CD8 T cell response and efficiently triggers production of IgG2a Abs. This is associated with Ag-specific protection in an aggressive model of melanoma. Systemic administration of free CpG however also induces non-Ag-specific, generalized immune activation. Injection of free CpG results in the rapid increase of systemic concentrations of proinflammatory cytokines and chemokines. These immune modulators trigger a wide range of secondary cellular effects such as activation of macrophages and monocytes, enhancement of neutrophil migration and reactive oxygen species production, NK cell activation, and production of proteins of the acute phase response (24, 25). Splenomegaly, disruption of lymphoid follicles and hepatic...
infiltrates associated with hepatotoxicity have also been described after repeated application of CpG in mice (18). In striking contrast to free CpG, nanoparticle-formulated CpG did not induce a generalized activation of the immune system. The release of inflammatory cytokines into the circulation, the early systemic activation of lymphocytes in the spleen and the later disruption of splenic microarchitecture were all abolished or strongly reduced by formulation of CpG with nanoparticles.

In humans, a rapid and highly sensitive systemic proinflammatory response following s.c. injection of free CpG is also observed, with increases in serum IL-6, IL12p40, and in products of IFN-inducible genes such as IP-10, MIG, MIP-Iβ, and MIP-3β (26, 27). Although this systemic induction of proinflammatory mediators may be beneficial for certain applications, it has also raised safety concerns. The rapid induction of proinflammatory cytokines can lead to the development of a systemic inflammatory response syndrome in humans, as has recently been described for another immunomodulator, TGN 1412 (28). CpG itself can cause a systemic inflammatory response syndrome in mice under certain conditions, such as after endotoxin priming (29). Clinical data on the long-term adverse effects of systemic TLR9 activation in humans, in particular concerning the enhancement of autoimmune responses, are not yet available. In an experimental setting, CpG treatment can lead to exacerbation of autoimmune disease in animal models of lupus, colitis, and arthritis (30–32). Furthermore, application of recombinant IFN-α, a major cytokine induced by free CpG, can lead to autoimmune disease in patients (33). Formulation of CpG with gelatin nanoparticles may therefore reduce the long-term risk of autoimmune reactions by abolishing unspecific immunostimulation and proinflammatory cytokine production while maintaining vaccine adjuvant efficacy.

Gelatin nanoparticle formulation modifies the distribution pattern of CpG in vivo. Free oligonucleotides given subcutaneously show high concentrations in the draining lymph nodes but also enter the systemic circulation and can be detected in the spleen, liver, and kidneys (34). In contrast, we show that CpG administered subcutaneously in nanoparticle formulation are detected almost exclusively in the draining lymph nodes. Their uptake by APCs results in direct cellular activation of the CpG-positive cell and triggers bystander activation of neighboring cells, leading to localized immune stimulation. Thus, CpG are targeted to the site of induction of the Ag-specific response. Indeed, induction of humoral and CDS T cell responses to Ag without systemic immune activation can also be achieved by direct intralymphatic administration of CpG (35). Furthermore, we show that CpG-bound nanoparticles inhibit the activation of two important immune suppressive mechanisms, up-regulation of the enzyme IDO and Treg cell-mediated suppression (19, 23).

Liposomal and other particulate delivery systems enhance the immunostimulatory activity of CpG, in particular with respect to IFN-α production (36, 37). The formulation of CpG results in longer retention in the endosomal vesicles, where the oligonucleotide binds to TLR9 which colocalizes with the downstream signaling complex MyD88-IRF-7. This results in sustained DC activation and IFN-α production (38). The enhanced cytokine production induced by formulated CpG is associated with an increase in adjuvant activity following immunization with Ag, especially when the Ag used is weak (39–42). Similarly, after internalization by APCs, gelatin nanoparticles also localized mainly to the endolysosomes (14). In contrast to other studies in which CpG was incorporated into the particles (39), the oligonucleotides used in this study were adsorbed onto the cell surface, thus ensuring that CpG is readily accessible to TLR9 binding in the endosome.

Gelatin nanoparticles may also serve as carrier for other types of oligonucleotides that are promising candidates for use as vaccine adjuvants. We have recently shown that single-stranded RNA oligonucleotides, a ligand for TLR7, efficiently trigger CD8 T cell responses to Ag (8). RNA oligonucleotides must however be applied using a delivery system to show immunostimulatory activity (3, 4, 9). Systemic application of these oligonucleotides, as is the case for CpG, also results in a unspecific and generalized activation of the immune system that may be suppressed by gelatin nanoparticle delivery (8). Furthermore, 5′-triphosphate RNA oligonucleotides, recognized by the cytoplasmic receptor RIG-I, induce strong IFN-α production and may serve as a vaccine adjuvant (5, 43). For this ligand also, a carrier to ensure delivery into the cell is required.

In conclusion, we show that gelatin nanoparticles are an efficient delivery system for CpG that selectively target the immunostimulatory activity to the draining lymph node. In the future, loading of immunostimulatory oligonucleotides together with Ag onto the same particles may further improve immunization outcome, as recent studies have shown that conjugation of TLR ligands and Ag results in superior activation of T cell responses (44). Thus, an all-in-one vaccine formulation in which gelatin nanoparticles simultaneously deliver Ag and adjuvant may represent an advantageous delivery system.

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Disclosures

The authors have no financial conflict of interest.

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