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Selectively targeting the toll-like receptor 9 (TLR9) – IRF 7 signaling pathway by polymer blend particles

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Abstract

Signaling through toll-like receptor 9 (TLR9) has been exploited for cancer therapy. The stimulation of TLR9 leads to two bifurcating signaling pathways – NF- κ B-dependent pro-inflammatory cytokines pathway and IRF-7-dependent type I interferons (IFNs) pathway. In this study, we employ polymer blend particles to present the synthetic ligand, CpG oligonucleotides (CpG ODNs) to TLR9. The polymer blend particles are made from the blend of pH-insensitive and pH-sensitive copolymer. By tailoring the composition of the pH-sensitive polymer, CpG ODNs are presented to TLR9 in a way that only activates the IRF-7 signaling pathway. CpG ODNs have been used for cancer therapy in both preclinical and clinical studies. The selective activation of IRF-7 could potentially enhance the apoptosis of tumor cells and immunological control of tumor progression without inadvertently activating NF- κ B-dependent oncogenesis.

Keywords

pH-sensitive polymer; carcinogenesis; inflammation; apoptosis; immunotherapy

1. Introduction

Signaling through Toll-like receptor 9 (TLR9) has been exploited for stimulating innate and adaptive immunity or intrinsic factors of infected and cancer cells for treating infectious diseases and cancers [1]. TLR9 was first identified as the receptor for bacterial DNA [2], which contains unmethylated cytosine-phosphate-guanine (CpG) motifs. Subsequently, several natural and synthetic TLR9 ligands have been identified, including DNA of viruses [3–5], fungi [6, 7], and dead cells [8, 9], as well as chromatin-IgG complexes [10, 11], dinucleotides [12] and CpG oligodeoxynucleotides (ODNs) [13]. In particular, CpG ODNs have demonstrated potential as vaccine adjuvants, microbicides, and anti-cancer drugs [14].

TLR9 resides in the endoplasmic reticulum. In order to signal, TLR9 has to be transported to and cleaved in endolysosomal compartments [15, 16]. Upon the ligation with its cognate ligands, TLR9 recruits the MyD88 adapter protein. Subsequently, two distinct pathways are initiated. One is the nuclear factor κ B (NF- κ B)–dependent pro-inflammatory cytokines pathway, and the other is the interferon regulatory factor 7 (IRF7)–dependent type I interferons (IFNs) pathway [17, 18]. NF- κ B is a family of transcription factors that control

many biological responses. It is well established that the activation of NF- κ B regulates immune responses and inflammation. Increasing evidence have shown that the unregulated activation of NF- κ B is linked to oncogenesis [19]. NF- κ B regulates the expression of proteins that promote cell proliferation and differentiation, migration and anti-apoptotic effects [20, 21]. The suppression of NF- κ B has been shown to be critical for the induction of apoptosis of a number of tumor cells such as multiple myeloma cells [22, 23], T cell lymphoma [24], prostate cancer cells [25] and breast cancer cells [26].

The activation of IRF-7 results in the production of type I IFNs (IFN- α and IFN- β) [17]. Type I IFNs play multiple roles in cancer therapy. They directly act on cancer cells and induce their apoptosis [27]. They also promote immunological control of cancer cells by increasing the number of tumor-antigen specific cytotoxic T cells [28, 29], programming dendritic cells (DCs) (i.e. enhancing the ability of cross presentation and priming of CD8⁺ T cells by CD8 α DCs [30]), and regulating functions of NK cells [31]. Recently, it has been shown that IRF-7 controls the metastasis of breast cancer cells. The down-regulation of IRF-7 leads to the dissemination of breast cancer cells to distal sites [32].

Clearly, the activation of the two arms of TLR9 signaling would have distinct consequences in cancer therapy by CpG ODNs or other ligands of TLR9. It is known that type B CpG ODNs activate the NF- κ B pathway only whereas type A CpG ODNs activate both NF- κ B and IRF-7 pathways [13]. In this study, we sought to design a biomaterial that would selectively activate the IRF-7 pathway while minimizing the activation of NF- κ B by type A CpG ODNs. Polymer blend particles were used to present type A CpG ODNs, i.e. CpG 2216, to TLR9. The polymer blend particles contained a pH-insensitive polymer, poly(lactic-co-glycolic acid) (PLGA) and a pH-sensitive copolymer. We hypothesized that varying the composition of the pH-sensitive copolymer could present type A CpG ODNs to TLR9 in a way that would not activate the NF- κ B pathway.

2. Materials and Methods

2.1. Cell culture

A dendritic cell line, BC-1 (a gift from Dr. Yoshiki Yanagawa), was maintained as described previously [33]. The single cell suspension of splenocytes was obtained from C57BL/6 mice (Jackson Laboratory). Spleens were collected and re-suspended in 0.5 ml of RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (complete media). Spleens were minced into small pieces. Tissues were incubated with 1.8 mg/ml collagenase D and 30 μ g/ml of DNase at 37 $^{\circ}$ C for 30 min. Tissues were centrifuged, the solutions discarded, and then re-suspended in Hank's Balance Salt Solution (HBSS) containing 5 mM EDTA and 1% FBS for 5 min at 37 $^{\circ}$ C. A single cell suspension was obtained by grinding the tissues through a 70 μ m cell strainer. The cells were incubated with ACK lysis buffer (0.15 M NH₄Cl, 1 M KHCO₃, and 0.1 mM Na₂EDTA, pH 7.4) to lyse red blood cells. Cells were washed, re-suspended in complete media and counted.

2.2. Reagents

ODNs, CpG 2216 and FITC-CpG 2216, were purchased from TriLink BioTechnologies (San Diego, CA). The sequence of CpG 2216 is 5' ggG GGA CGA TCG TCg ggg gG3'. Lowercase letters denote a phosphorothioate backbone, and uppercase letters denote a phosphodiester backbone. FITC-CpG 2216 was synthesized by labeling the ODN with FITC at the 5' end. Antibodies used for immunocytochemistry were purchased from Abcam and Invitrogen. Antibodies used for ELISA were purchased from eBioscience, PBL InterferonSource, Cell Sciences, and Jackson ImmunoResearch. All cell culture reagents

were from Life Technologies, NY. Tetrahydrofuran (THF) and dichloromethane (DCM) were supplied by EMD Chemicals Inc., NJ. Diethyl propylmalonate, butyl methacrylate, 2-(dimethylamino)ethyl methacrylate, 2,2'-azobis(2-methylpropionitrile) (AIBN) and poly(vinyl alcohol) (PVA) were purchased from Sigma-Aldrich. Poly(lactic-co-glycolic acid) (PLGA, 50:50, IV=0.65dL/g) was from LACTEL (DURECT Corporation, AL).

2.3. Synthesis of pH-sensitive polymers

The free radical polymerization of PAA, BMA and DMAEMA was performed by following a procedure reported previously [34]. The PAA monomer was synthesized by following a protocol adapted from a method reported previously [35]. PAA, BMA and DMAEMA were vacuum distilled before use. Monomers (PAA:DMAEMA:BMA=1:1:2, DMAEMA:BMA=1:1 or DMAEMA:BMA=1:4 (mole ratio)) and AIBN (2 mM) were mixed in THF (monomer:THF = 10 wt%). The polymerization was carried out at 60 °C for 15 h (terpolymer) or 80 °C for 20 h (dipolymer) under nitrogen. The polymer was precipitated in an excess amount of diethyl ether and pentane and dried under vacuum. These three polymers (BMA-PAA-DMAEMA (terpolymer), BMA-DMAEMA (1:1) (1:1 dipolymer) and BMA-DMAEMA (3:1) (3:1 dipolymer)) were characterized by proton nuclear magnetic resonance (^1H NMR) spectroscopy.

2.4. Fabrication of polymer blend particles

The blend particles were fabricated by using the double emulsion method. The mixture of PLGA and pH-sensitive polymer (terpolymer, 1:1 dipolymer or 1:3 dipolymer) mixture was dissolved in 1 ml of DCM with a weight ratio of 4:1 (PLGA: pH-sensitive polymer) overnight. HiLyte647-labeled PLGA (10 wt% in total polymer) was also doped into the mixture of the polymers in order to fluorescently label the particles. FITC-CpG 2216 or CpG 2216 was dissolved in 100 μl of Dulbecco's Phosphate-Buffered Saline (DPBS) to different concentrations (10 or 5 mg/ml, respectively), and the CpG 2216 solution was added to the polymer solution drop-wise. 2 ml of 5% PVA was then added drop-wise into the polymer solution while vortexing, and the solution was sonicated and paused for 10 seconds alternately. The emulsion was then added to 4 ml of 5% PVA under stirring and followed by the sonication as described before. Then the emulsion was poured into 4 ml of 0.06% PVA solution under stirring, and the final emulsion was stirred for 3 to 4 h at room temperature to completely evaporate DCM and allow blend particles to harden. The particles were washed 3 times with Milli-Q water and stored in DPBS at 4°C. The particles were named as Terpolymer, 1:1 Dipolymer and 3:1 Dipolymer depending on the pH-sensitive polymer used, and Terpolymer – CpG, Dipolymer 1:1 – CpG or Dipolymer 3:1 – CpG if CpG ODNs were incorporated.

2.5. Characterization of blend particles

A Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) was used to characterize the size distribution by dynamic light scattering (DLS) and the zeta potential of the particles. Particles were prepared at 0.05 mg particle/ml for DLS measurements. Size and zeta potential measurements were performed at room temperature in 10 mM of KNO_3 at different pHs from 4 to 9. To determine the surface charge of the particles at physiological temperature and pH, zeta potential measurements were also performed at 37 °C in a buffer composed of 10 mM citric acid and 20 mM disodium hydrogen phosphate at the pH ranging from 4.57 to 7.30. SEM and TEM were used to determine the particle size and examine the morphology of the particles. SEM samples of particles were spin-coated onto silica, sputter-coated with 12 nm of platinum using a SPI Sputter Coater (Structure Probe, Inc., West Chester, PA), and analyzed using a Siron SEM with a beam voltage of 5.0 kV (NTUF, University of Washington). For TEM samples, 6 μl of particle solution was added onto 400 mesh copper TEM grids coated with continuous carbon film. After 2 minutes, the TEM grid

was blotted with Whatman filter paper from the side and dried in air. TEM samples were examined by a Tecnai TF20 transmission electron microscope (FEI) at 200 kV. Electron images were recorded on a $4k \times 4k$ CCD camera (Gatan, Inc., CA) at 50,000 magnification (effective pixel size of 0.4 nm) and approximately -1 mm defocus. The electron dose for each exposure was approximately $20 \text{ e}/\text{\AA}^2$.

2.6. Quantification of the polymer composition and the loading of CpG 2216 in blend particles

For NMR spectroscopy, blend particles were dissolved in deuterated methylene chloride and samples were analyzed using a ^1H Bruker AV500 spectrometer. Using the hydrogens on the ester groups of PLGA (5.2 ppm) and the side chain of BMA (3.9 ppm), and the known ratios of the monomers of the polymers, the fraction of terpolymer in blend particles was calculated.

CpG 2216-containing particles were diluted in 0.05 ml DPBS and centrifuged at 13,200 rpm for 10 min. The supernatant was collected. The pellet was dissolved in 0.5 ml of 1% (w/w) sodium dodecyl sulfate (SDS)/0.1 M NaOH, and subsequently incubated in a water bath at 90°C for 5 min to ensure the complete dissolution of particles. In parallel, a known concentration of soluble FITC-CpG 2216 prepared in 1% (w/w) SDS/0.1 M NaOH and subjected to heating as samples was used for preparing standard curve. It was confirmed that incubation at 90°C did not affect the fluorescent properties of FITC-CpG. SpectraMax (Molecular Devices) was used to quantify the fluorescence of FITC-CpG 2216 at excitation and emission wavelengths of 494 and 520 nm, respectively. The amount of CpG 2216 in the supernatant and pellet was quantified by using the standard curve.

2.7. Quantification of FITC-CpG 2216 released from particles at 37°C at different pHs

To determine the release of CpG 2216 from particles, particles were incubated in buffer solutions that mimicked the pH and temperature that endocytosed particles were exposed to. The buffer solutions were composed of 10 mM citric acid and 20 mM disodium hydrogen phosphate at pH 4.57 to 7.30. Particles were diluted in the buffer solutions at room temperature, and aliquoted at 0.1 ml ($50 \mu\text{g}$ particles) per micro-centrifuge tube. Next, the aliquots were immersed in a water bath at 37°C . At the given time point, an aliquot was centrifuged at 13,200 rpm for 10 min at room temperature. The supernatant was collected and the pellet was dissolved as described above to completely release FITC-CpG 2216. The FITC-CpG 2216 in the supernatant and pellet was quantified according to the procedure stated above.

2.8. Preparation and characterization of CpG 2216-adsorbed particles

Fluorescent polystyrene (PS) particles (Polysciences, Inc., Warrington, PA) of 100 nm in diameter were coated with CpG 2216 in our previous work [33]. Briefly, 100 nm PS particles were coated with poly-L-lysine (PLL; Sigma-Aldrich, Inc., Saint Louis, MO; MW = 30,000–70,000 g/mol). Next, CpG 2216 (TriLink BioTechnologies, San Diego, CA) was adsorbed onto PLL-coated particles, since PLL is a cationic polymer and able to facilitate the adsorption of CpG 2216. CpG 2216-adsorbed particles are denoted as 2216-PLL-PS. We have previously characterized the cytokine profile of BC-1 cells stimulated with 2216-PLL-PS [33]. 2216-PLL-PS was used as a positive control in this study.

Terpolymer-coated CpG 2216 particles were fabricated by preparing blank terpolymer particles in 10 mM KNO_3 at pH 2.5, and CpG 2216 in 10 mM KNO_3 at pH 2.5. Next, 0.25 ml of particles was added drop-wise to 0.25 ml of CpG while vortexing after adding each drop. The final concentrations in the mixture were 0.66 mg terpolymer particle/ml and 0.15

mg CpG/ml. The adsorption reaction proceeded for 2 h at room temperature. Particles were washed three times in 10 mM KNO₃ at pH 6 by centrifugation at 2000 rpm for 30 min.

2.9. Stimulation of cells by CpG 2216

BC-1 cells were seeded at a density of 2.5×10^5 cells per well in a 24-well plate. Cells were incubated at 37 °C overnight to allow cells to adhere to the plate surface. CpG 2216-loaded blend particles, 2216-PLL-PS, and soluble FITC-CpG 2216 and CpG 2216 (0.005 mg/ml) were added to cells in 0.5 ml of medium. After 24 h of incubation at 37 °C, cell supernatants were collected and stored at -20 °C for the measurement of cytokines.

1×10^6 splenocytes in 0.1 ml medium were added to a well of a 96-well round-bottom plate. Next, 0.1 ml of CpG 2216-containing samples were added to the cells so that the final volume was 0.2 ml per well. After 24 h of incubation at 37 °C, cell supernatants were collected and stored at -20 °C for the measurement of cytokines.

2.10. Measurement of endosomal pH

The endosomal pH was measured by using a fluorescent ratiometric method that was adapted from a previously established method [36]. For cells used for standard curves, BC-1 cells were seeded at a density of 3×10^6 cells per well in a 6-well plate, and incubated overnight at 37 °C. Cells were subsequently incubated with particles for 3 h (pulse), washed and further incubated for an additional 4 h (chase). Particles contained pH-sensitive FITC-labeled CpG 2216 and pH-insensitive HiLyte 647-labeled PLGA. Afterwards, cells were harvested and processed to construct a standard curve. The cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 2 min at room temperature, and resuspended in 0.1 M citrate buffer solutions of known pH values. Citrate buffer solutions were prepared in DPBS. For samples in which endosomal pH was measured, cells were seeded at 2.5×10^5 cells per well, and incubated overnight at 37 °C. The pulse and chase condition was the same as above. Cells were harvested and resuspended in different concentrations of NH₄Cl in Isocove's Modified Dulbecco's Medium (IMDM) supplemented with 0.5% FBS and incubated for 1 h at 37 °C. NH₄Cl was used to adjust endosomal pH as in our previous study [33]. Cell suspensions were analyzed using a FACSCanto (Cell Analysis Facility, University of Washington). The geometric mean fluorescence intensity (gMFI) of cells was determined by FlowJo (Treestar, Inc., Ashland, OR). The ratio of gMFI of FITC to that of HiLyte 647 was compared to the standard curve constructed in citrate buffers with known pH values to obtain the endosomal pH of each sample.

2.11. Measurement of cytokine concentrations

Cytokine concentrations in cell supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA). The procedures for assaying IL-6 were adapted from the manufacturer's protocol (eBioscience, San Diego, CA). The procedures for IFN- α were described previously [4, 37].

2.12. Immunocytochemistry

BC-1 cells were plated at a density of 2.5×10^5 cells per well in 500 μ l medium onto 24-well plates. Cells were allowed to adhere to the surface overnight at 37 °C. Cells were pulsed with particles at 100 μ g particles/ml in 500 μ l medium for 3.5 h at 37 °C. Then, particles were removed and cells were washed with DPBS. Cells were detached from the tissue-culture surface by trypsinization and then plated onto 12 mm circular glass coverslips (Erie Scientific Co., Portsmouth, NH). To enhance the adherence of cells to coverslips, coverslips were first pre-treated with poly-D-lysine (PDL, Sigma-Aldrich, Inc., Saint Louis, MO) by

incubating them with 400 μ l PDL at 0.025 mg/ml for 30 min at room temperature. Coverslips were washed three times with 500 μ l Milli-Q water and allowed to dry before the seeding of cells. Cells were allowed to adhere for 20 min. Afterward, cells were fixed and permeabilized with 250 μ l of BD Cytofix/Cytoperm™ (Invitrogen, Frederick, MD) for 20 min at 4 °C. Cells were washed twice with 250 μ l of blocking buffer. Blocking buffer contained 1 wt% bovine serum albumin (Sigma-Aldrich, Inc., Saint Louis, MO) in BD Perm/Wash Buffer™ (Invitrogen, Frederick, MD). Next, cells were blocked with the blocking buffer for 1.5 h at room temperature. To examine p65 and IRF7, cells were labeled with 250 μ l blocking buffer containing either 2 μ g/ml rabbit polyclonal anti-p65 (Abcam, Cambridge, MA) or 2 μ g/ml rabbit polyclonal anti-IRF7 (Abcam, Cambridge, MA) for 1 h at room temperature. Cells were washed twice with the blocking buffer. To detect primary antibodies, cells were incubated with 250 μ l of blocking buffer containing 2 μ g/ml Cy3-anti rabbit IgG (Abcam, Cambridge, MA). Lastly, cells were washed twice with the blocking buffer. Coverslips were rinsed with Milli-Q water and then mounted onto glass slides using DAPI-containing HardSet Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA) for confocal microscopic analysis.

2.13. Confocal microscopy and image analysis

Images were acquired with a Zeiss LSM 510 META confocal microscope equipped with a 63×1.40 numerical aperture (N.A.) PLAN APO oil immersion differential interference contrast objective lens (Keck Imaging Center, University of Washington). All images were acquired by using the line sequential scanning mode (one channel is scanned at a time). Images were acquired for single-stained samples to confirm that crosstalk between channels did not exist. The cross-reactivity of the secondary antibody was examined and determined to be undetectable.

Nuclei were approximately 1 μ m thick in the z direction. The middle optical section of the nucleus was used to determine whether a significant amount of p65 or IRF-7 was present in the nuclei. Each optical section was 0.9 μ m thick, so the middle optical section of nuclei represented a significant portion of a nucleus.

2.14. Statistical analysis

All experiments were repeated two to three times. The one-tailed and unpaired Student's *t* test was used to analyze the differences between experimental groups as specified. $p < 0.05$ is considered as statistically different between two experimental groups.

3. Results and Discussion

3.1. Characterization of pH-sensitive copolymers

The pH-sensitive copolymers used for particle fabrication were composed of all three or two of the following monomers, 2-propylacrylic acid (PAA), 2-(dimethylamino)ethyl methacrylate (DMAEMA) and butyl methacrylate (BMA) (Fig. 1). They were synthesized by radical polymerization with a number-averaged MW of 20 kDa. BMA facilitates the mixing of pH-sensitive copolymers with PLGA to form the particles. The PAA and DMAEMA monomers have pK_a of 6.7 and 8.3, respectively [38, 39]. Both monomers are protonated, and undergo conformational changes at the endosomal/lysosomal pH [40]. The copolymer that consisted of all three monomers is termed as terpolymer, and of two monomers (DMAEMA and BMA) as dipolymer throughout the text. Based on proton nuclear magnetic resonance (1H NMR) analysis (Fig. S1), the molar ratio of monomers in the terpolymer was 3:2:5 (PAA:DMAEMA:BMA). The dipolymers were composed of either 1:1 (dipolymer 1:1) or 3:1 (dipolymer 3:1) of BMA to DMAEMA.

3.2. Composition, size, morphology, and surface charge of polymer blend particles

Blend particles were made from the mixture of pH-sensitive copolymers and PLGA by the double-emulsion method [41]. The type A CpG ODN, CpG 2216, was incorporated into the particles. These particles are denoted as Terpolymer, Dipolymer 1:1 or Dipolymer 3:1 depending on which pH-sensitive copolymer was used. Terpolymer blend particles onto which CpG 2216 was adsorbed are called Terpolymer-adsorb CpG. The composition of polymers in the blend particles was determined by ^1H NMR. The BMA peak (3.9 ppm) and PLGA peak (5.3 ppm) were used to calculate the actual polymer ratio in the blend particles (Fig. S1 and Table 1). All three pH-sensitive polymers and CpG 2216 were successfully incorporated into blend particles. The amount of CpG 2216 in each type of blend particle was nearly the same.

The hydrodynamic diameters of polymer blend particles were between 150 to 250 nm at pH 7.0 ± 0.1 (Fig. 2A). They were nearly spherical as examined by scanning electron microscopy (SEM) (Fig. 2C) and transmission electron microscopy (TEM) (Fig. 2D). The micrographs of TEM also revealed that blend particles appeared as core/shell structures. pH-sensitive copolymers contained hydrophilic side chains. They likely remained at the interface of aqueous and organic phases during the particle fabrication process, resulting in two distinct phases in blend particles. The negatively charged CpG ODNs were expected to interact with the positively charged DMAEMA and increase the tendency of phase separation of the two polymers in the blend particles.

The zeta potential of polymer blend particles was measured at different pHs (Fig. 2B). The control particles (Terpolymer-adsorb CpG), onto which negatively charged CpG ODNs are adsorbed, had a strong negative charge (-35 mV) as expected. Blend particles with (Terpolymer-CpG) and without CpG 2216 (Terpolymer) had a strong positive surface charge at an acidic pH (23 and 32 mV, respectively), which can be attributed to the tertiary amine in the DMAEMA monomer. The zeta potential of both types of particles decreased as the pH reached neutral and basic conditions due to the deprotonation of PAA and DMAEMA. Terpolymer-CpG exhibited a lower zeta potential than Terpolymer within the range of pHs examined, indicating a fraction of the negatively-charged CpG ODNs were present on the surface of particles. The zeta potential of particles made from the dipolymer exhibited different trends. Dipolymer 1:1 (particles without CpG 2216) exhibited positive surface charge between pH 4 and 9, indicating the dipolymer 1:1 was present on the surface of particles. In contrast, the zeta potential of Dipolymer 1:1-CpG displayed a higher positive charge below pH 7 and a lower positive charge above pH 7. This indicates that CpG 2216 was still present on the surface, but the positive charge from the protonated DMAEMA dominated over the negatively charged CpG 2216 when pH was below 7. The zeta potential of Dipolymer 3:1-CpG was approximately -30 mV between pH 5 and 9 and approached to neutral at pH 4 while that of Dipolymer 3:1 (particles without CpG) were nearly neutral between pH 5 and 9 and increased up to 5 mV at pH 4. This result suggests that CpG 2216 was present on the surface of Dipolymer 3:1 and dominated over the positive charge from DMAEMA between pH 5 and 9. In comparison with particles made from the dipolymer 1:1, it indicates that the increasing ratio of BMA resulted in less DMAEMA in dipolymers to be present on the surface of particles. In summary, CpG 2216 was present on the surface of all the types of blend particles. By comparing the zeta potential of particles with and without CpG 2216 at pH 9.0, it can be estimated that the amount of CpG 2216 was on the surface of particles in the order of Terpolymer $\sim$ Dipolymer 3:1 $>$ Dipolymer 1:1.

3.3. Cytokine profiles induced by CpG2216-containing polymer blend particles

CpG 2216 is a type A CpG motif-containing ODN, which is known to induce both pro-inflammatory cytokines (i.e. IL-6, IL-12, TNF- α) through the activation of NF- κ B and type

I IFNs, such as IFN- α , through the activation of IRF-7 [13]. The cytokine secretions induced by different CpG 2216-containing or -coated particles were assessed by using splenocytes isolated from mice. CpG 2216 was labeled with fluorescein (FITC) for the ease of quantification of CpG 2216 in particles. When CpG 2216 was incorporated into the dipolymer 1:1 (Dipolymer 1:1-CpG), negligible amounts of IL-6 and IFN- α were secreted. When the amount of DMAEMA in the dipolymer was reduced to a molar ratio of BMA to DMAEMA of 3:1 (Dipolymer 3:1-CpG), both IL-6 and IFN- α were secreted. When CpG 2216 was incorporated into the terpolymer particles (Terpolymer-CpG), only IFN- α secretions could be detected – the pro-inflammatory cytokines, such as IL-6, nearly diminished within the wide range of concentrations of CpG 2216 (Fig. 3A). Interestingly, both IL-6 and IFN- α were secreted upon the stimulation by terpolymer particles onto which CpG 2216 were directly adsorbed (Terpolymer-adsorb CpG), suggesting the incorporation of CpG 2216 into particles – which may result in a unique interaction between CpG 2216 with TLR9 – was critical for selectively targeting the IRF-7 pathway. Terpolymer particles loaded with non-fluorescent CpG 2216 exhibited similar results, indicating that the modification of CpG 2216 by FITC did not contribute to the observed trend (Fig. S2).

These results indicate that cytokine profiles can be tuned through the presentation of CpG ODNs by polymer blend particles. Encouraged by these results, we subsequently focused on the Terpolymer-CpG particle only. For convenience and the humane use of mice, the dendritic cell line, BC-1 cells [42], was used for all subsequent studies. We confirmed that the cytokine profiles of BC-1 cells stimulated with Terpolymer-CpG consisted of IFN- α , but not IL-6 secretions as observed for splenocytes from mice (Fig. 3B). Cytokine secretions from controls, such as soluble CpG 2216, and CpG 2216-adsorbed polystyrene (CpG-PLL-PS), were expected and consistent with previous reports [33].

3.4. Impaired translocation of NF- κ B by Terpolymer-CpG

IL-6 is one of the pro-inflammatory cytokines whose induction is regulated by the transcription factor, NF- κ B, while IFN- α , is regulated by the transcription factor, IRF-7, upon the ligation of TLR9 by CpG ODNs. In resting cells, NF- κ B complexes are sequestered in the cytoplasm. Upon their activation, they enter the nuclei and bind to the target gene. To confirm Terpolymer-CpG was not able to activate the NF- κ B complexes, we then investigated the translocation of p65, one of the subunits of the NF- κ B complex, as well as the translocation of IRF-7 from the cytoplasm to the nucleus through immunofluorescence (Fig. 4). The amount of p65 that was present in the nuclei of cells treated with Terpolymer-CpG was similar to those treated with Terpolymer or no particles, but significantly lower than those treated with Terpolymer-adsorb CpG particles. IRF-7 was present at significantly higher amounts in the nuclei of cells treated with Terpolymer-CpG and Terpolymer-adsorbed CpG particles than in the nuclei of cells treated with Terpolymer or no particles. We did notice that the overall level of IRF-7 was reduced in cells treated with Terpolymer-CpG in comparison to cells treated with Terpolymer-adsorb CpG particles. We speculate that the level of IRF-7 is regulated by NF- κ B as suggested by previous studies [43–45]. Our results so far suggest that Terpolymer-CpG is able to regulate TLR9 signaling by selectively activating the IRF-7 pathway, but not the NF- κ B pathway.

3.5. Mechanisms of selectively targeting the IRF-7 pathway by Terpolymer-CpG

Next, we attempted to understand the mechanisms by which Terpolymer-CpG selectively targeted the IRF-7 pathway. Endosomal pH is known to affect TLR9 signaling. The recruitment of TLR9 to endocytic compartments is pH-dependent, and the interaction between TLR9 and CpG ODNs requires the acidification of endosomes [15, 46]. Our previous studies have also shown that pro-inflammatory cytokines are induced when TLR9 signaling occurred in a more basic environment (pH > 6.0) while the induction of type I

IFNs required further acidification of endosomal compartments. First, we measured the pH of endosomal compartments containing Terpolymer-CpG. After a 3 h pulse and 4 h chase, the endosomal pH was 5.08 and 4.82 for 50 and 100 $\mu\text{g/ml}$ of Terpolymer-CpG particles, respectively (Fig. 5A). We hypothesized that it was possible that Terpolymer-CpG was quickly routed to an acidic environment and did not permit the sufficient interaction between TLR9 and CpG ODNs in a more basic environment for the activation of NF- κ B. We were not able to measure endosomal pH at short durations. Instead, we used NH_4Cl to buffer the acidification of endosomes as we did previously [33]. We tested a range of concentrations of NH_4Cl (Fig. 5B). At higher than 1 mM NH_4Cl , CpG 2216 adsorbed on PS beads (CpG-PLL-PS) did not induce any IL-6, indicating that the recruitment of TLR9 or the interaction of TLR9 with CpG ODNs at a more basic environment was compromised. We decided to use 1 mM of NH_4Cl to test whether raising the pH could rescue the signaling of TLR9 for the generation of IL-6. The presence of 1 mM of NH_4Cl raised the endosomal pH by 0.20 and 0.24 pH units for 100 and 50 μg of Terpolymer-CpG particle/ml, respectively (Fig. 5A), and the IFN- α response induced by Terpolymer-CpG significantly reduced (Fig. 5B) as expected. Still, no IL-6 was detected. In contrast, in the presence of NH_4Cl at 1 mM, CpG 2216-coated PS beads (2216-PLL-PS) induced less IFN- α , but IL-6 production was unaffected (Fig. 5B), consistent with our previous observations [33]. These results demonstrate that the retention of Terpolymer-CpG in a less acidic environment did not promote the interaction of CpG 2216 with TLR9 for the induction of pro-inflammatory cytokines.

We then measured the quantity of CpG 2216 that was released from Terpolymer-CpG under conditions that mimicked the pH and temperature that endocytosed particles were exposed to. Surprisingly, throughout the period of 24 h, the amount of CpG 2216 that was released from particles was negligible at pH 4.57 to 6.11 (Fig. 5C), which is the pH range of endocytic compartments. At pH 7.30, the pH of the extracellular environment, approximately 5% of the incorporated CpG was released from the particles. This indicated that most of the CpG was retained in the particles prior to and after particle internalization. The release of CpG 2216 at different pH environments was unlikely the factor for selectively targeting the IRF-7 pathway.

CpG 2216 was retained in particles within a range of pH 4.57 to 6.11 (Fig. 5C). Then, we tested whether CpG 2216 was present on the surface of Terpolymer-CpG within the pH range of endocytic compartments. We measured the surface charge of Terpolymer-CpG and Terpolymer (particles without CpG ODNs) and compared them to determine whether CpG 2216 was exposed at the particle surface at pHs between 5 and 7 (Fig. 5D). In the terpolymer, the two functional units in PAA and DMAEMA have a pK_a of 6.7 and 8.3, respectively [38, 39]. The pK_a of monomers was expected to change within a polymer matrix. Nearly all of the DMAEMA was expected to be ionized within the pH range of endosomal compartments as suggested by the zeta potential change of Dipolymer 1:1 (Fig. 2B), and 50% of PAA to be ionized at pH 6.5 as suggested by the zeta potential change of blend particles containing a dipolymer of BMA and PAA (1:1) (Data not shown). Ionized DMAEMA endowed the terpolymer particles with a positively charged surface (and positive zeta potential) while ionized PAA yielded a negative charge. The zeta potential of Terpolymer particles (without CpG 2216) between pH 4.57 and 6.11 decreased from 13 to 7 mV, indicating more and more PAA was de-ionized and counteracted the positive charge of DMAEMA. The CpG 2216 was negatively charged and expected to counteract the positive charge of the terpolymer as well. Between pH 7.30 and 4.57, the surface charge of Terpolymer-CpG was less than that of Terpolymer particles, which suggested that a portion of CpG 2216 was displayed on the surface of particles at the pH of all of the endocytic compartments.

Then, we asked whether the terpolymer would affect the interaction of binding between TLR9 and CpG 2216 at different pHs. The TLR9 structure has not been resolved yet. But the structure of TLR3, which binds to double stranded RNA (dsRNA), has been resolved recently [47, 48]. It has been suggested that the phosphodiester backbone, rather than the base sequence of the RNA, contributes to critical interactions. Histidines are the key residues of TLR3 that interact with dsRNA [49, 50]. The binding of TLR9 to ssDNA is suggested to be similar to that of TLR3 to dsRNA [51]. At a more basic pH, more PAA was ionized and negatively charged, which might compete with the interaction between CpG 2216 and TLR9. This is also consistent with our observation that Dipolymer 3:1, which only consisted of DMAEMA, was able to stimulate IL-6. At pH 5.53 and 4.57, the majority of PAA was de-ionized, which left more opportunities for CpG 2216 to interact with TLR9 for the activation of the IRF-7 pathway. So far, we do not have the direct evidence to prove the above speculation. Detailed structural studies, surface analysis and the survey of a library of different compositions of Terpolymer (such as varying ratios of PAA and DMAEMA) will elucidate mechanisms of selectively targeting TLR9 signaling pathways by Terpolymer-CpG blend particles.

4. Conclusion

Our results have demonstrated the use of polymer blend particles for the controlled activation of TLR9 signaling pathways. Terpolymer blend particles exhibit the unique ability to present type A CpG ODNs to TLR9 and induce IRF-7 activation only. In the context of cancer immunotherapy, the selective activation of IRF-7 pathway could potentially enhance the apoptosis of tumor cells and immunological control of tumor progression without inadvertently activating NF- κ B-dependent oncogenesis. Broadly, blend particles offer an excellent biomaterial tool for finely tailoring both innate and adaptive immunity instigated through TLR9 signaling, and for understanding molecular and cellular mechanisms underlying the bifurcation of TLR9 signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Chemical structure of PLGA, Terpolymer, Dipolymer 1:1 and Dipolymer 3:1

Fig. 2. Size, zeta potential, and morphology of polymer blend particles

(A) The number average of hydrodynamic diameter and (B) zeta potential of polymer blend particles suspended in 10 mM KNO₃ at designated pHs. (C) SEM and (D) TEM images of polymer blend particles.

Fig. 3. Effect of the composition of the pH-sensitive polymer on the cytokine secretions of cells
The levels of IL-6 and IFN-α from (A) splenocytes and (B) BC-1 cells after 24 h incubation with the indicated stimulants. 5 µg/ml of soluble CpG 2216 was used. The * indicates that the cytokine level was undetectable.

Fig. 4. Effect of Terpolymer-CpG particles on the nuclear translocation of transcription factors Particles were incubated with BC-1 cells for 4 h at 37 °C followed by a 3 h chase. Next, cells were stained with either rabbit anti-p65 or rabbit anti-IRF7, followed by the detection antibody, Cy3-goat anti-rabbit IgG (red). Nuclei were stained with DAPI (blue). 11 ~ 35 cells in total were examined for each sample.

Fig. 5. Mechanisms of selectively targeting IRF-7 pathway by Terpolymer-CpG particles
 (A) The pH of particle-containing endosomes 6 h after cells were exposed to Terpolymer-CpG at 50 and 100 μg particle/ml. (B) The level of IL-6 and IFN- α secreted by BC-1 cells after 24 h-incubation with Terpolymer-CpG and CpG-PLL-PS. 5 $\mu\text{g/ml}$ of soluble CpG 2216 was used. The * symbol indicates that the cytokine level was undetectable. (C) Effect of pH on the release profile of CpG 2216 from Terpolymer-CpG. Particles were incubated in 10 mM citric acid and 20 mM disodium hydrogen phosphate at 37 °C. "Pellet" indicates the amount of CpG 2216 in particles, and "sup" indicates the CpG 2216 released from particles. (D) Effect of pH on the zeta potential (surface charge) of Terpolymer-CpG. Particles were incubated in 10 mM citric acid and 20 mM disodium hydrogen phosphate at 37 °C. The surface charge of Terpolymer-CpG and Terpolymer were measured at 37 °C. " $\text{ZP}_{\text{Ter}} - \text{ZP}_{\text{Ter-CpG}}$ " is the difference between the surface charge of Terpolymer and Terpolymer-CpG.

Table 1

The polymer compositions and CpG 2216 in blend particles.

Particle name	Copolymer used in fabrication (wt. fraction)	Actual copolymer in particle (wt. fraction)	mg CpG 2216 per mg particle
Terpolymer-CpG	0.2	0.15	0.041
Terpolymer	0.2	0.24	0
Dipolymer 1:1-CpG	0.2	0.11	0.037
Dipolymer 1:1	0.2	0.11	0
Dipolymer 3:1-CpG	0.2	0.21	0.031
Dipolymer 3:1	0.2	0.32	0