

Ferumoxytol and CpG oligodeoxynucleotide 2395 synergistically enhance antitumor activity of macrophages against NSCLC with EGFR^{L858R/T790M} mutation

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Purpose: Drug resistance is a major challenge for epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) treatment of lung cancer. Ferumoxytol (FMT) drives macrophage (MΦ) transformation towards a M1-like phenotype and thereby inhibits tumor growth. CpG oligodeoxynucleotide 2395 (CpG), a toll-like receptor 9 (TLR9) agonist, is an effective therapeutic agent to induce anticancer immune responses. Herein, the effect of co-administered FMT and CpG on MΦ activation for treating non-small cell lung cancer (NSCLC) was explored.

Methods: The mRNA expression levels of M1-like genes in RAW 264.7 MΦ cells stimulated by FMT, CpG and FMT and CpG (FMT/CpG) were evaluated by quantitative reverse transcription PCR (qRT-PCR). Then, the effects of FMT/CpG-pretreated MΦ supernatant on apoptosis and proliferation of H1975 cells were detected by flow cytometry, and the expression of EGFR and its downstream signaling pathway in H1975 cells were explored by western blotting. Finally, a H1975 cell xenograft mouse model was used to study the anti-tumor effect of the combination of FMT and CpG in vivo.

Results: FMT and CpG synergistically enhanced M1-like gene expression in MΦ, including tumor necrosis factor-α, interleukin (IL)-12, IL-1α, IL-1β, IL-6 and inducible nitric oxide synthase (iNOS). FMT/CpG-pretreated MΦ supernatant inhibited proliferation and induced apoptosis of H1975 cells, accompanied by down-regulation of cell cycle-associated proteins and up-regulation of apoptosis-related proteins. Further studies indicated that the FMT/CpG-pretreated MΦ supernatant suppressed p-EGFR and its downstream AKT/mammalian target of rapamycin signaling pathway in H1975 cells. Furthermore, FMT/CpG suppressed tumor growth in mice accompanied by a decline in the EGFR-positive tumor cell fraction and increased M1 phenotype macrophage infiltration.

Conclusion: FMT acted synergistically with CpG to activate MΦ for suppressed proliferation and promoted apoptosis of NSCLC cells via EGFR signaling. Thus, combining FMT and CpG is an effective strategy for the treatment of NSCLC with EGFR^{L858R/T790M} mutation.

Keywords: ferumoxytol, CpG oligodeoxynucleotide, macrophages, non-small cell lung cancer, epidermal growth factor receptor

Introduction

The incidence of lung cancer has been increasing yearly with a mortality rate ranking first among malignant tumors.¹ EGFR-tyrosine kinase inhibitors (TKIs) are effective for the treatment of lung cancer patients with EGFR mutation,²⁻⁴ however, drug

resistance develops in most cases after 9–13 months.⁵ Although molecular targeted therapy improves patient survival as compared to conventional treatment approaches such as radiotherapy, chemotherapy, and surgery, the 5-year overall survival rate is still <15%.⁶ Therefore, there is an urgent need to develop alternative treatment strategies for non-small cell lung cancer (NSCLC) harboring resistance mutations.

Immunotherapy has shown great potential for cancer treatment.^{7–9} Macrophages (M Φ) are heterogeneous immunocytes accounting for a significant proportion in the tumor microenvironment (TME).¹⁰ Activated M Φ can be divided into two subtypes according to function:^{11,12} M1, which mediates inflammation and the anti-tumor immune response by producing pro-inflammatory factors such as tumor necrosis factor (TNF)- α and IL-12; and M2, which secretes high levels of immunosuppressive mediators to promote tumor growth, invasion, and metastasis. NSCLC is associated with greater infiltration of M Φ than other cancers¹³ and the density of M2 M Φ was negatively correlated with the survival of lung cancer patients who had undergone surgery.¹⁴ In addition, M2 M Φ infiltration reduces the sensitivity of EGFR-TKIs in lung adenocarcinoma.^{15,16} Whereas gefitinib inhibits M2-like polarization of M Φ in Lewis lung cancer,¹⁷ which represents a promising approach in modulating the TME for lung cancer treatment. However, although restoring the immunological activity of M Φ has substantial antitumor effects, treatment of NSCLC with EGFR mutation—particularly T790M—by steering M Φ to M1 phenotype has not been reported.

Ferumoxyl (FMT) is a versatile nanoparticle that has been widely used as a drug carrier.^{18–21} However, its potential as an immune-activator to treat tumors has been overlooked. A recent study has showed that iron released by lysed red blood cells induces the conversion of M Φ into a pro-inflammatory phenotype capable of directly killing tumor cells; besides, an abundant of iron-loaded M Φ is correlated with reduced tumor size in NSCLC patients.¹³ In addition, FMT synergized with TLR3 agonist poly (I:C) significantly suppressed the growth of melanoma by shifting macrophages to a tumoricidal phenotype in our previous work.²² This suggests that activation of M Φ by FMT may be an effective strategy for the treatment of lung cancer, although it has not been investigated in NSCLC with EGFR^{T790M} mutation.

CpG oligodeoxynucleotide 2395 (CpG) is an artificially synthesized oligodeoxynucleotide containing unmethylated CpG motifs that triggers a pro-inflammatory immune response by interacting with Toll-like receptor (TLR) 9 in M Φ .^{23,24} The antitumor effect of CpG has been

demonstrated in several malignancies including stage I/II melanoma,²⁵ glioblastoma,^{26,27} and non-Hodgkin's lymphoma.²⁸ However, not all clinical studies on CpG reported the improved patient outcomes. For instance, CpG did not enhance the therapeutic effect of erlotinib in patients with advanced recurrent EGFR-positive NSCLC.²⁹ Whereas, on the other hand, CpG in combination with the first-line drug taxane and platinum chemotherapy prolonged survival in patients with advanced NSCLC³⁰ indicating that CpG may potentially maximize the benefits of immunotherapy in patients with EGFR^{T790M} mutation by combined therapy.

In this study, we investigated the above possibility in the present study using the human NSCLC cell line H1975 with EGFR^{L858R/T790M} mutations and a xenograft mouse model. Although treatment with FMT and CpG alone or in combination did not have an inhibitory effect on tumor cells, the supernatant of M Φ pretreated with both FMT and CpG (FMT/CpG) promoted apoptosis and inhibited proliferation in tumor cells by suppressing the expression of EGFR signaling pathway components. These results provide the first demonstration that in addition to traditional chemotherapy and molecular targeted therapy, M Φ activation by FMT/CpG is an effective strategy for the treatment of NSCLC with EGFR^{L858R/T790M} mutations.

Materials and methods

Cell culture and reagents

The TLR9 agonist CpG (class C ODN 2395) was purchased from InvivoGen (San Diego, CA, USA; #tlrl-2395–5). FMT was a gift from Prof. Ning Gu.^{31,32} The RAW 264.7 M Φ cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). H1975 cells with EGFR^{L858R/T790M} mutation were provided by Prof. Yongqian Shu (The First Affiliated Hospital of Nanjing Medical University, Nanjing, China) and the use of H1975 cells are approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University for the experimental study. The cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific) at 37°C in a humidified atmosphere of 5% CO₂.

The cells were passaged at 70–80% confluence. RAW 264.7 cells were seeded in 24-well plates and FMT (100 μ g/mL) and CpG (2.5 μ g/mL) were added to the medium; the M Φ supernatant was collected after stimulation for

12 h. H1975 cells were seeded in a 24-well plate. When the cells were attached, the supernatant was discarded and the cells were treated with FMT (100 µg/mL), CpG (2.5 µg/mL), or the supernatant of MΦ grown for 12 hrs with or without FMT/CpG. H1975 cells were cultured for 48 hrs at 37°C in a 5% CO₂ incubator and used for cell proliferation, apoptosis, and Western blotting experiments.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from harvested cells using TRIzol reagent (Thermo Fisher Scientific; #15596018). The mRNA was reverse transcribed into cDNA using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd, Nanjing, China; #R211) according to the instructions. QRT-PCR was performed in SYBR Green PCR Master Mix (Thermo Fisher Scientific; #4309155) on a Step One Plus system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The relative expression levels of target genes were calculated with the $2^{-\Delta\Delta Ct}$ method relative to the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences are shown in Table 1.

Cell viability assay

The effect of FMT and CpG on NSCLC progression was examined using Cell Counting Kit (CCK)-8 (Dojindo Laboratories, Kumamoto, Japan; #CK04). The cells were seeded at a density of 5×10^3 /well in 96-well plates and incubated overnight at 37°C in an atmosphere of 5% CO₂. The cells were then treated with fresh medium containing different concentrations of FMT, CpG, and FMT/CpG for 48 h. The supernatant was discarded and replaced with fresh medium containing 10% CCK-8 solution, followed by incubation for 1–4 h. The absorbance at 450 nm was measured using a multi-well spectrophotometer (BioTek, Winooski, VT, USA). There were six replicate wells for each sample and the experiment was repeated three times.

Flow cytometry (FCM) analysis of cell apoptosis

Cell apoptosis was detected by FCM using the Annexin V-Alexa Fluor 488/PI Apoptosis Assay kit (FcMACS, Nanjing, China; #FMSAV488-100). H1975 cells were collected, washed twice in phosphate-buffered saline at 4°C, and resuspended in binding buffer. A 100-µL of the cell suspension was transferred to a flow tube, and 5 µL

Table 1 Sequences of forward and reverse primers used for qRT-PCR

Gene	Gene ID	Primer sequence
GAPDH	14,433	5'-TGACCTCAACTACATGGTCTACA-3'
		5'-CTTCCCATTCTCGGCCTTG-3'
TNF-α	21,926	5'-CAGGCGGTGCCTATGTCTC-3'
		5'-CGATCACCCGAAGTTCAGTAG-3'
IL-12p40	16,160	5'-GTCCTCAGAAGCTAACCATCTCC-3'
		5'-CCAGAGCCTATGACTCCATGTC-3'
IL-1α	16,175	5'-AGTATCAGCAACGTCAGCAA-3'
		5'-TCCAGATCATGGGTATGGACTG-3'
IL-1β	16,176	5'-GAAATGCCACCTTTTGACAGTG-3'
		5'-TGGATGCTCTCATCAGGACAG-3'
IL-6	16,193	5'-CTGCAAGAGACTTCCATCCAG-3'
		5'-AGTGGTATAGACAGGTCTGTTGG-3'
CD86	12,524	5'-TCAATGGGACTGCATATCTGCC-3'
		5'-GCCAAAATACTACCAGCTCACT-3'
iNOS	18,126	5'-ACATCGACCCGTCCACAGTAT-3'
		5'-CAGAGGGGTAGCTTGTCTC-3'

Abbreviations: CD86, cluster of differentiation 86; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; qRT-PCR, quantitative reverse transcription-PCR; TNF-α, tumor necrosis factor-α.

Annexin V-Alexa Fluor 488 and 10 µL propidium iodide (PI) were added, followed by mixing and incubation at 4°C for 15 mins in the dark. The cell suspension was sorted on a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA). Early and late apoptotic cell fractions (Annexin V-positive and Annexin V/PI double-positive, respectively) were quantified.

FCM analysis of cell proliferation

H1975 cells were labeled with 10 µM 5(6)-carboxyfluorescein diacetate N-hydroxysuccinimidyl ester (CFSE; Thermo Fisher Scientific; #65-0850-84) and incubated in CFSE staining solution for 15 mins at 37°C. An equal volume of culture medium (containing serum) was added to the cells along with CFSE staining solution, followed by incubation for 5 mins. The CFSE-containing solution was removed and the cells were washed twice with an equal volume of culture medium. The fluorescently labeled cells were used for subsequent experiments. Cell proliferation was detected by FCM. The proliferation index was calculated using ModFit LT software (Verity Software

House, Topsham, ME, USA) as the total number of divisions divided by the number of proliferating parent cells.^{33,34}

Colony formation assay

H1975 cells were seeded at a density of 1×10^3 /well in 12-well plates and incubated overnight at 37°C in an atmosphere of 5% CO₂. The supernatant was discarded, and the cells were cultured for 2 weeks under different treatment conditions. The medium was refreshed at appropriate intervals, which was determined according to the pH of the culture supernatant. The experiment was terminated when macroscopic clones appeared in the culture plates, which were then washed twice with PBS, fixed in 4% formalin for 10–15 mins, dried, and stained with 0.1% crystal violet for 15 mins. The number of colonies was counted under a microscope and those with >50 cells were defined as positive.

Western blotting

Cells subjected to different treatments were lysed in ice-cold radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China; #P0013K), and protein concentration was determined using a bicinchoninic acid protein quantitation kit (Vazyme Biotech Co., Ltd; #E112-01). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad) that was blocked in 5% bovine serum albumin for 1 hrs at room temperature and then incubated overnight at 4°C with primary antibodies. After washing three times with Tris-buffered saline with 0.1% Tween 20, the membrane was incubated with secondary antibody (Thermo Fisher Scientific; #A16110SAMPLE; #A16078SAMPLE) for 1 hrs at room temperature. Protein expression was visualized by enhanced chemiluminescence Western blotting detection reagent (Millipore, Billerica, MA, USA; #WBULS0500), and protein band intensity was quantified using ImageJ software. Primary antibodies against B-cell lymphoma-2-associated X protein (Bax; #5023), Cleaved Caspase-3 (Asp175; #9661), Cleaved poly(ADP ribose) polymerase (PARP, Asp214; #5625), Cyclin A2 (#4656), Cyclin B1 (#12231), EGFR (#4267), phospho-EGFR (p-EGFR, Tyr1068; #3777), AKT (#4691), p-AKT (Ser473; #4060), mammalian target of rapamycin (mTOR; #2983), p-mTOR (Ser2448; #5536) and GAPDH (#5174) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Tumor xenograft studies

H1975 cells were resuspended in 100 µL sterile PBS, and 2×10^6 cells were subcutaneously injected into the right flank of 5–6 weeks old female BALB/c nude mice (Shanghai Laboratory Animal Research Center, Shanghai, China). When the tumors reached a volume of 100 mm³, the animals were randomly divided into two groups ($n=5$ each) that were intratumorally injected with PBS or FMT (10 mg/kg) and CpG (2.5 mg/kg) every 2 days for 10 days. Body weight, maximum tumor length (L), and minimum tumor width (W) were recorded every 3 days. The tumor volume was calculated with the following formula: $V = (L \times W^2)/2$. At the end of the experiment, the mice were euthanized and the tumors were excised, washed with PBS, and fixed in formalin for immunohistochemistry. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Nanjing medical University and conformed to the Guidelines for the Care and Use of Laboratory Animals.

Immunohistochemistry

Tumor tissue specimens were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 µm-thick sections that were deparaffinized with xylene, rehydrated in a graded series of ethanol for 5 mins, washed three times with PBS, and then blocked with serum for 30 mins. The sections were incubated overnight at 4°C with primary antibody, washed three times with PBS, incubated with biotinylated secondary antibody for 30 mins at 37°C, and then washed with PBS, followed by staining with 3,3'-diaminobenzidine at room temperature for 10 mins in the dark. After staining with hematoxylin for 2 mins, the sections were subjected to hydrochloric acid/alcohol differentiation, dehydrated with ethanol and xylene, dried, and photographed under a microscope.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc, Chicago, IL, USA). Data were compared by one-way ANOVA or Student's *t*-test. All statistical analyses were conducted at the significant level of $\alpha=0.05$ and the Least Significance Difference or Dunnett's test were used for post hoc of ANOVA analysis.

Results

Characterization of FMT

The polymer coating the outer layer of FMT was synthesized by terminal aldehyde group reduction and

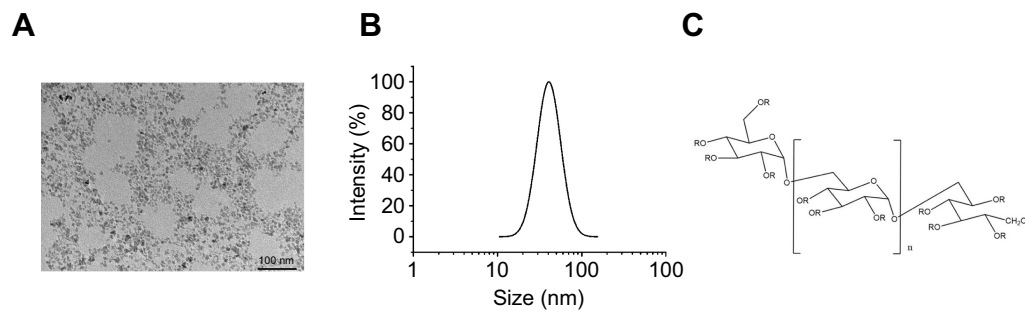


Figure 1 Characterization of FMT. **(A)** Transmission electron micrograph of FMT. **(B)** Hydration particle size of FMT was shown by dynamic light scattering. **(C)** Molecular formula of polymer compound coating the outer layer of FMT, where the R group is H or COOH. **Abbreviations:** COOH, carboxyl; FMT, ferumoxytol.

hydroxycarboxymethylation of dextran T10. As indicated in [Figure 1A](#), the average diameter of synthesized dextran T10-coated FMT was about 7 nm and dynamic light scattering showed that the hydration particle size of FMT was 35 nm ([Figure 1B](#)). The molecular formula of the FMT external material is shown in [Figure 1C](#), with H or COOH as the R group.^{31,32}

FMT and CpG synergistically promote M1-like gene expression in MΦ

To investigate the effects of FMT, CpG, and FMT/CpG on MΦ activation, the mRNA expression levels of M1-like genes in

RAW 264.7 cells stimulated for 12 hrs were examined by qRT-PCR. FMT/CpG synergistically enhanced the expression of the M1-like genes of TNF- α , IL-12, IL-1 α , IL-1 β and IL-6 compared to either agent alone or the lipopolysaccharide (LPS)-stimulated positive control group ([Figure 2](#)). Among these altered genes, IL-12 was upregulated to the greatest degree following co-stimulation ([Figure 2B](#)), with a transcript level that was 109 times higher than that in control MΦ. FMT or CpG treatment alone had a similar albeit less potent effect (15- and 36-fold higher expression, respectively, as compared to the control. In contrast, in MΦ stimulated with LPS as a positive control, IL-12 mRNA expression was increased by only 20 fold relative to the control. In

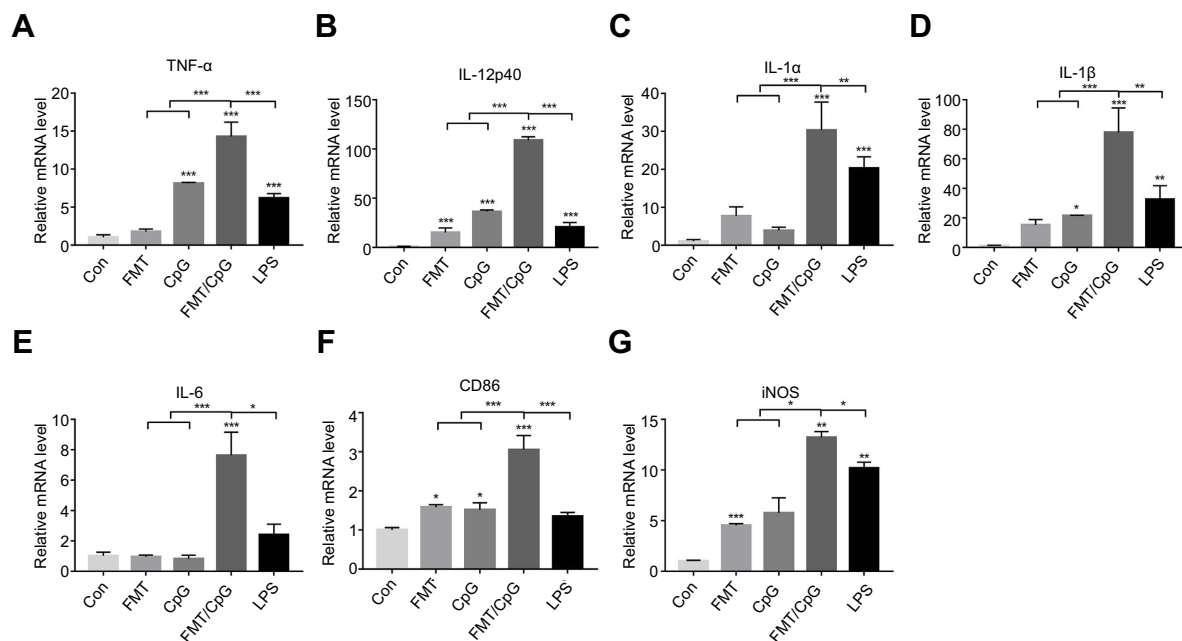


Figure 2 FMT synergizes with CpG ODN 2395 to promote M1-like gene expression in MΦ. Relative mRNA expression was analyzed by qRT-PCR in RAW 264.7 cells following treatment with FMT (100 μ g/mL), CpG (2.5 μ g/mL), both FMT and CpG (FMT/CpG), or LPS (100 ng/mL) for 12 hrs. Results are expressed as mean \pm SD of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 vs control.

Abbreviations: FMT, ferumoxytol; LPS, lipopolysaccharide; MΦ, macrophages; ODN, oligodeoxynucleotide; qRT-PCR, quantitative reverse transcription PCR.

addition, the mRNA level of the M1-related co-stimulatory molecule cluster of differentiation 86 (CD86) and inducible nitric oxide synthase (iNOS) was also enhanced by FMT/CpG as compared to treatment with each agent alone. Thus, FMT and CpG synergistically promote M Φ activation towards a tumoricidal phenotype, with upregulation of M1-like genes.

FMT/CpG-pretreated M Φ supernatant reduce NSCLC cell viability

To determine whether FMT and CpG directly suppress NSCLC growth, H1975 cells were treated with FMT, CpG, and a combination of both for 48 hrs. FMT showed no obvious toxicity to H1975 cells (Figure 3A). CpG reduced cell viability in a dose-dependent manner, although the effect was non-significant at low doses (Figure 3B). In addition, the rate of inhibition upon treatment with FMT combined with CpG was only 13% (Figure 3C). These results demonstrate that FMT/CpG shows negligible cytotoxicity towards H1975 cells.

As demonstrated previously, co-treatment with FMT and CpG synergistically induced M Φ activation towards a tumoricidal phenotype. To investigate whether this phenotypic switch can lead to inhibition of tumor cell growth, H1975 cells were treated with FMT/CpG, the supernatant of M Φ grown for 12 hrs without any stimulation (-FMT/CpG M Φ S), or the supernatant of M Φ pretreated with FMT/CpG for 12 hrs (+FMT/CpG M Φ S). After 48 hrs, cell viability was detected. As shown in the Figure 3C, the cell viability of H1975 exposed to +FMT/CpG M Φ S was only 58.9% compared with the control group, while that in the -FMT/CpG M Φ S group was 80.48%. Altogether, FMT/CpG-pretreated M Φ supernatant has a significant inhibitory effect on H1975 cell viability.

FMT/CpG-pretreated M Φ supernatant induces apoptosis of NSCLC cells

The decrease in cell viability of H1975 may be a combined effect of tumor cell apoptosis and inhibited proliferation. First, H1975 cells were treated with FMT/CpG, -FMT/CpG M Φ S or +FMT/CpG M Φ S for 48 hrs and cell apoptosis was analyzed by FCM. The results of Figure 4A and B showed that FMT/CpG and -FMT/CpG M Φ S induced a low level of apoptosis in H1975 cells (9.18% and 10.6%, respectively). In comparison, the rate of apoptosis was significantly higher in cells exposed to FMT/CpG-pretreated M Φ supernatant than in control cells (46.2% vs 5.01%). To investigate the mechanism underlying this effect, we examined the expression of several apoptosis-related proteins by Western blotting and found that FMT/CpG-pretreated M Φ supernatant increased the expression levels of the apoptosis-related proteins Bax, Cleaved Caspase-3, and Cleaved PARP in H1975 cells (Figure 4C and D).

FMT/CpG-pretreated M Φ supernatant suppresses NSCLC cell proliferation

Next, the proliferation of H1975 cells pre-labeled with CFSE and subjected to different treatments was analyzed by FCM. As indicated in Figure 5A and B, the proliferation rate of H1975 cells in the control group was retarded in the fourth generation, with an average proliferation index of 7.2. There was no change in the index of H1975 cells treated with FMT/CpG. The proportion of cells in the fourth generation decreased upon treatment with -FMT/CpG M Φ S, yielding an average proliferation index of 5.62 while the proliferation of H1975 cells in the +FMT/CpG M Φ S group was markedly blocked in the third generation, with a proliferation index of

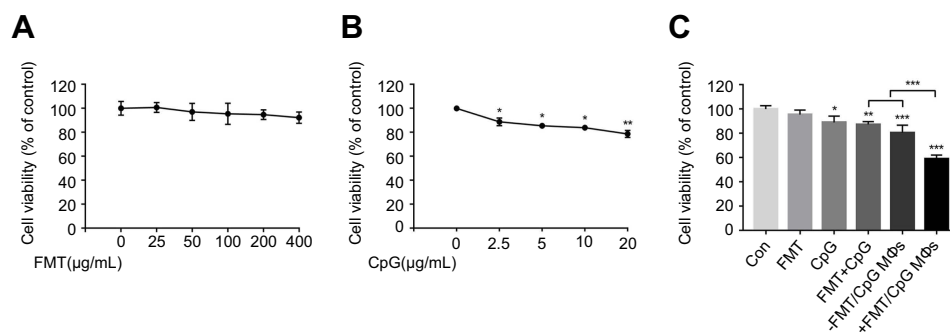


Figure 3 FMT/CpG-pretreated M Φ supernatant reduces H1975 cell viability. (A and B) H1975 cells were incubated with different concentrations of FMT (0–400 μ g/mL) or CpG (0–20 μ g/mL) for 48 hrs and cell viability was evaluated with the CCK-8 assay. (C) Effect of FMT (100 μ g/mL), CpG (2.5 μ g/mL), FMT/CpG, the supernatant of M Φ grown for 12 hrs without stimulation (-FMT/CpG M Φ S), or supernatant of M Φ pretreated with FMT/CpG for 12 hrs (+FMT/CpG M Φ S) on the viability of H1975 cells after 48 hrs. Results are expressed as mean \pm SD of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 vs control.

Abbreviations: CCK-8, Cell Counting Kit-8; FMT, ferumoxytol; M Φ , macrophages.

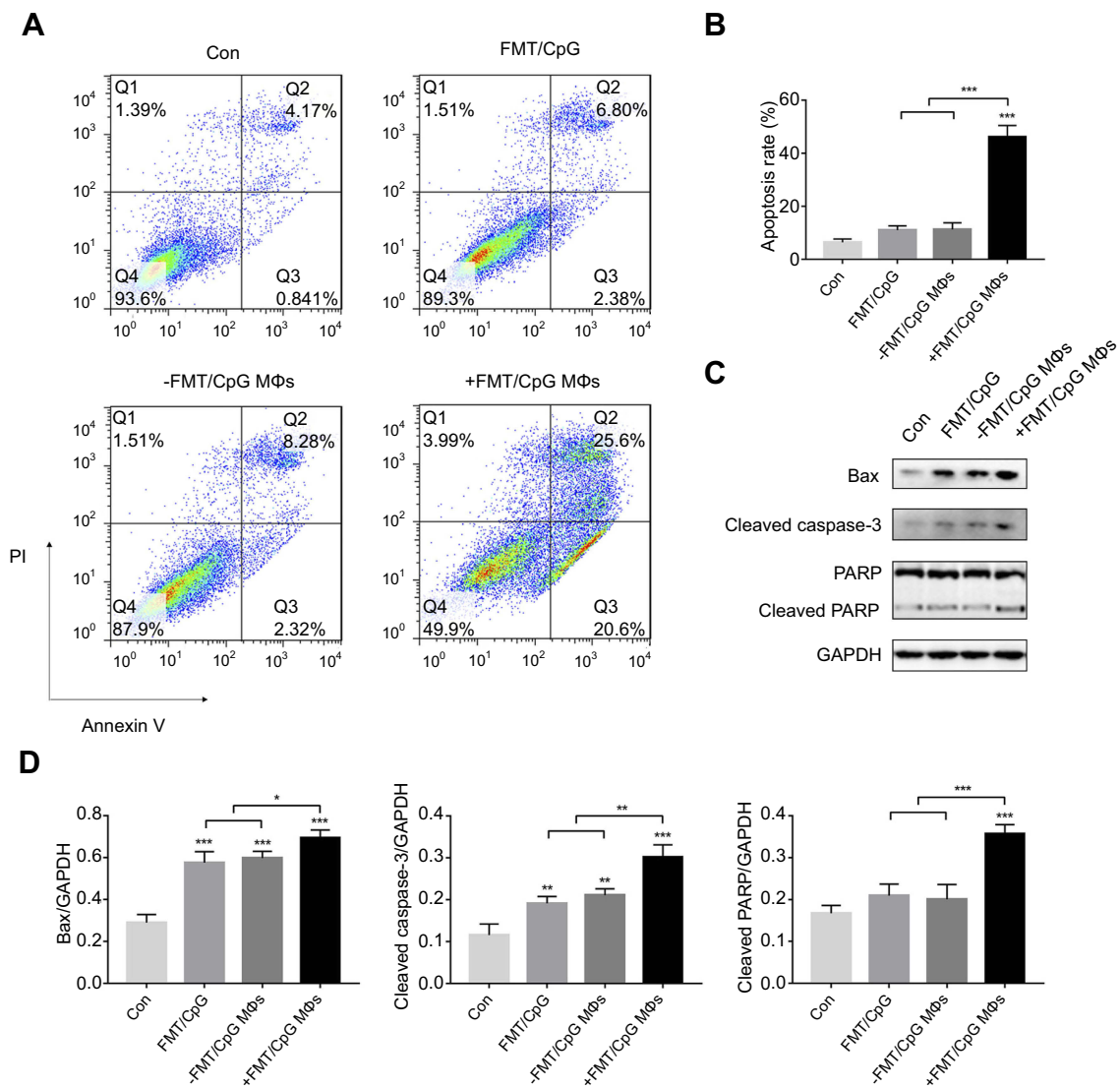


Figure 4 FMT/CpG-pretreated MΦ supernatant induces apoptosis of H1975 cells. **(A)** H1975 cells were treated with FMT/CpG, -FMT/CpG MΦs or +FMT/CpG MΦs. After 48 hrs, cells were stained with Annexin V/PI and cell apoptosis was analyzed by FCM. **(B)** Quantitative analysis of apoptotic H1975 cells. **(C)** Protein levels of Bax, Cleaved Caspase-3, and Cleaved PARP were evaluated by Western blotting in H1975 cells treated as indicated for 48 hrs. **(D)** Protein levels were quantified with ImageJ software and normalized to that of GAPDH. Results are expressed as mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

Abbreviations: Bax, B-cell lymphoma-2-associated X protein; FCM, flow cytometry; FMT, ferumoxytol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MΦ, macrophages; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide.

4.5, indicating that FMT/CpG-pretreated MΦ supernatant suppressed NSCLC cell growth. In addition, we verified the effect of +FMT/CpG MΦs on H1975 cell proliferation and clump development by colony formation assay. The results showed that FMT/CpG-pretreated MΦ supernatant significantly inhibited tumor growth and interfered development of tumor-like cell clumps (Figure 5C and D). A Western blotting analysis further revealed that the cell cycle-associated S/G2 phase regulatory protein Cyclin A2 and the G2/M phase regulatory protein Cyclin B1 were both downregulated to a greater extent in H1975 cells treated with +FMT/CpG MΦs as compared to the other three groups (Figure 5E and F), suggesting that the

cells were arrested in the S^{35,36} and G2/M^{37,38} phases of the cell cycle. Collectively, these results confirm that FMT/CpG-pretreated MΦ supernatant inhibits the proliferation of H1975 cells.

FMT/CpG-pretreated MΦ supernatant downregulates p-EGFR and its downstream AKT/mTOR signaling in H1975 cells

Activation of EGFR and its downstream AKT-mTOR signaling pathway leads to increased proliferation and

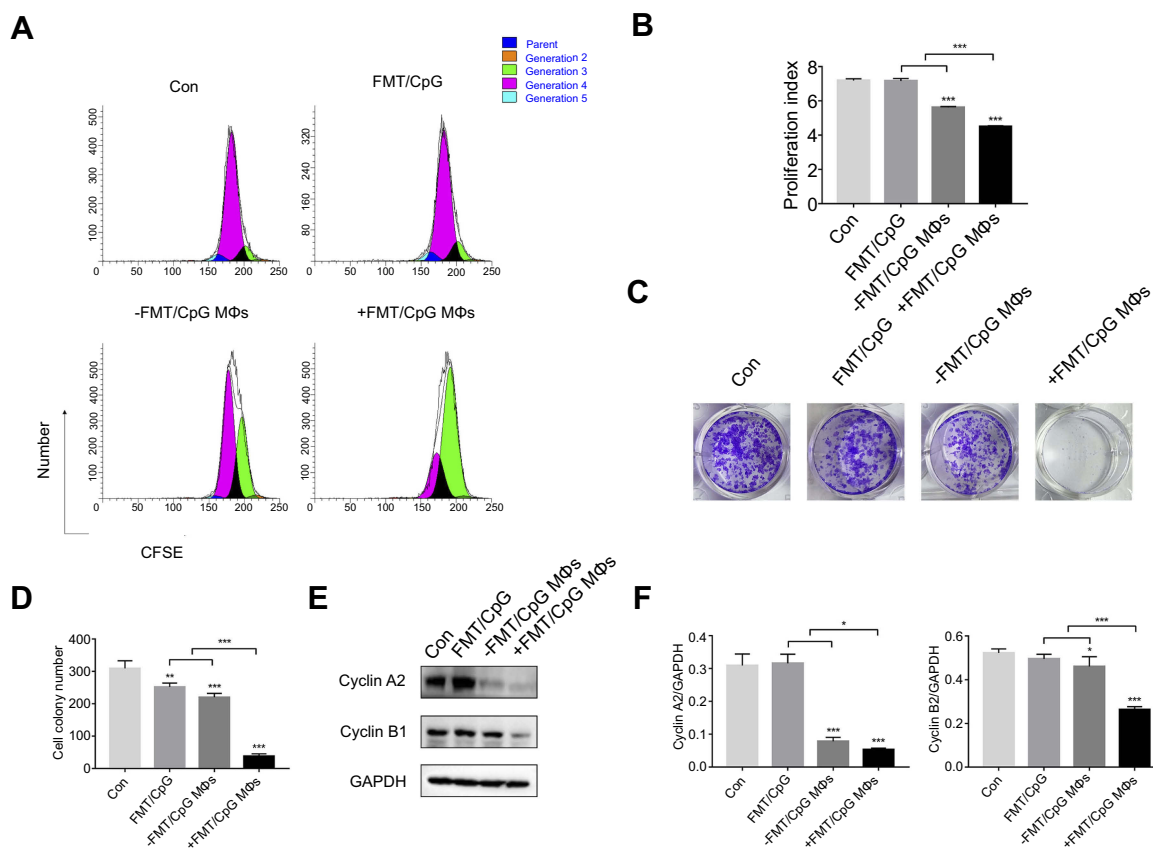


Figure 5 FMT/CpG-pretreated MΦ supernatant inhibits the proliferation of H1975 cells. **(A)** H1975 cells labeled with CFSE were subjected to indicated treatments for 48 hrs, and the proliferative fraction was analyzed by FCM. **(B)** Proliferation index of H1975 cells treated as indicated was determined with ModFit LT software. **(C and D)** Colony formation of H1975 cells following indicated treatments was quantified and the colonies of >50 cells were defined as positive. **(E)** Protein levels of Cyclin A2 and Cyclin B1 were detected by Western blotting analysis of H1975 cells following indicated treatments for 48 hrs. **(F)** Protein levels were quantified with ImageJ software and normalized to that of GAPDH. Results are expressed as mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

Abbreviations: CFSE, 5(6)-carboxyfluorescein diacetate N-hydroxysuccinimidyl ester; FCM, flow cytometry; FMT, ferumoxytol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MΦ, macrophages.

decreased apoptosis of tumor cells.³⁹ Next, Western blotting was used to detect whether the FMT/CpG pretreated macrophage supernatant affects the expression of EGFR and its downstream signaling pathway proteins in H1975 cells. As shown in Figure 6A and B, in contrast to the control group, the expression levels of p-EGFR, p-AKT, and p-mTOR proteins in H1975 cells treated with -FMT/CpG MΦs were partially inhibited, but not as potently as levels of the +FMT/CpG MΦs group. Compared to the FMT/CpG group, incubated with FMT/CpG pre-treated macrophage supernatant more considerably downregulated the levels of above proteins. Collectively, the results indicated that FMT/CpG pre-treated macrophage supernatant significantly promoted cell apoptosis and inhibited cell proliferation by downregulating phosphorylation of EGFR and its downstream AKT/mTOR protein in H1975 cells.

FMT and CpG synergistically inhibit tumor growth in a xenograft mouse model

We next investigated whether the combination of FMT and CpG has anti-tumorigenic effects in vivo using an H1975 cell xenograft mouse model. As showed in Figure 7A and B, co-administration of FMT and CpG significantly suppressed the tumor growth in tumor-bearing mice. Ki-67, an antigen present in the nuclei of proliferating cells, is widely used to evaluate the proliferative activity of tumors.⁴⁰ The immunohistochemical analysis revealed that the percentage of Ki-67- and EGFR-positive tumor cells in the FMT/CpG group was markedly lower than that in the control group (Figure 7D and E), whereas the percentage of M1 macrophages stained with F4/80 and iNOS in tumor tissues was significantly up-regulated

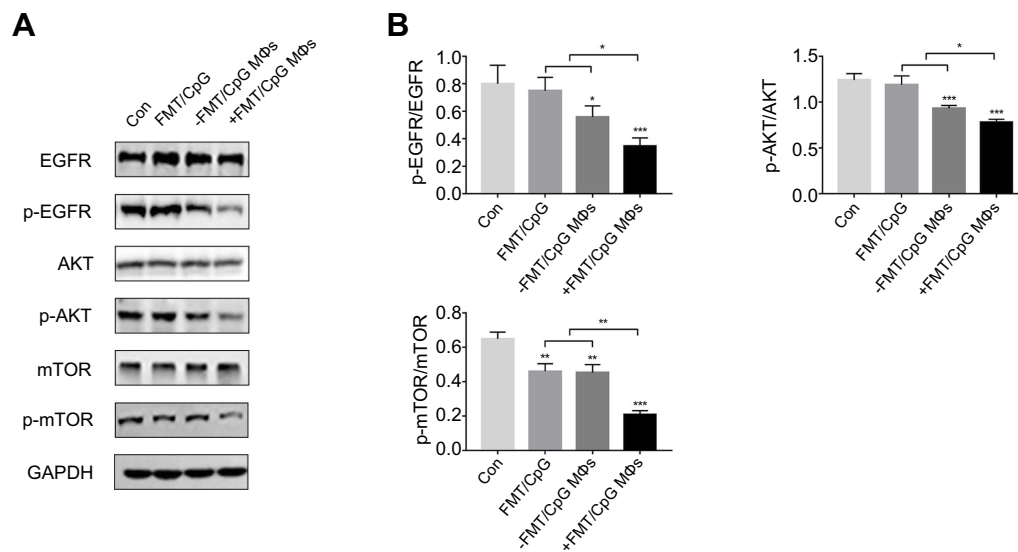


Figure 6 FMT/CpG pretreated M Φ supernatant suppresses the expression of EGFR and downstream signaling in H1975 cells. **(A)** H1975 cells were treated as indicated for 48 hrs, and protein levels of p-EGFR and its down p-AKT and p-mTOR were analyzed by Western blotting. **(B)** Protein levels were quantified with ImageJ software and phosphorylated protein levels were normalized to total protein levels. Results are expressed as mean \pm SD of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 vs control.

Abbreviations: AKT, protein kinase B; EGFR, epidermal growth factor receptor; FMT, ferumoxytol; M Φ , macrophages; mTOR, mammalian target of rapamycin.

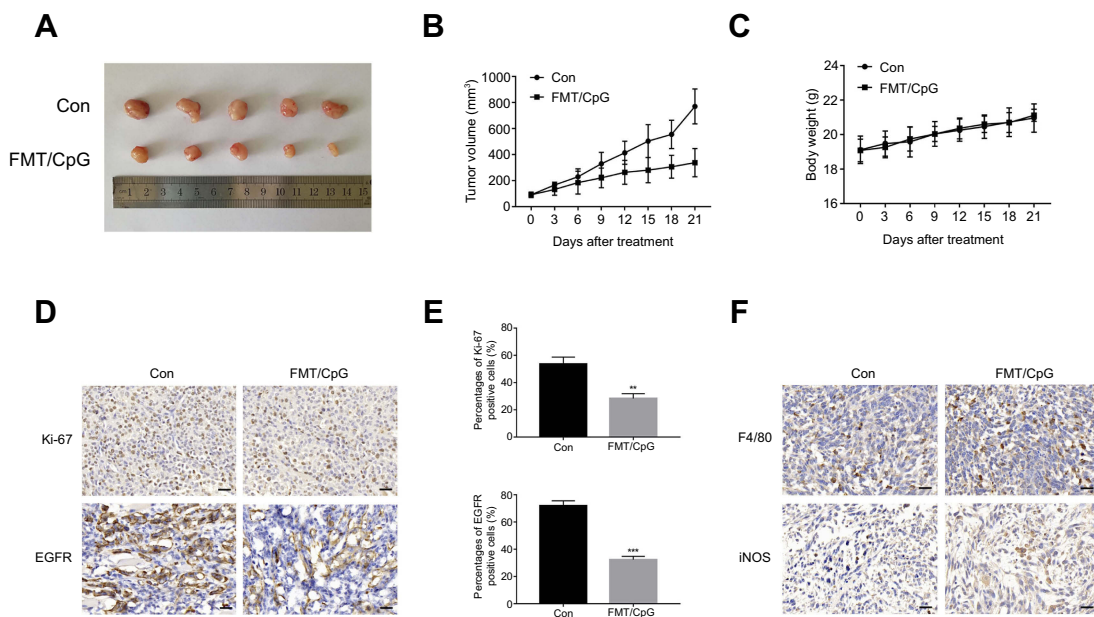


Figure 7 FMT and CpG synergistically inhibit tumor growth in a nude mouse xenograft model. **(A)** Image of tumors harvested at the end of treatment. **(B)** Tumor growth curves of tumor-bearing mice. **(C)** Body weight of tumor-bearing mice during the experiment. **(D)** Ki-67 and EGFR expression of tumor cells detected by immunohistochemistry. Scale bar: 50 μ m. **(E)** Quantitative analysis of **(D)**. **(F)** Macrophages stained with F4/80 and iNOS in tumor tissues detected by immunohistochemistry. Scale bar: 50 μ m. Results are expressed as mean \pm SD of three independent experiments. ** P <0.01, *** P <0.001 vs control.

Abbreviation: EGFR, epidermal growth factor receptor; FMT, ferumoxytol.

(Figure 7F). In addition, the mice showed no obvious weight loss or treatment-related death, indicating that the combined treatment was non-toxic (Figure 7C). Thus, FMT acts synergistically with CpG to suppress tumor growth in vivo.

Discussion

Osimertinib, a third-generation EGFR-TKI that has been approved for the treatment of EGFR^{T790M}-positive NSCLC patients, acts by inhibiting p-EGFR and downstream signaling.⁴¹ However, with changes in the TME

and the continuous transformation of cancer cells, the emergence of new drug-resistant mutations is inevitable.⁴² Various strategies have been developed to overcome resistance to EGFR-TKI treatment including combination chemotherapy,⁴³ antitumor vascular therapy,⁴⁴ and immunotherapy based on inhibitors of programmed cell death-1 and its ligand.⁴⁵ However, the toxicity of standard drugs and the high cost of the latter two approaches limit their universal application. As such, there is an urgent need for safer, more effective, and affordable therapeutic strategies to improve the outcome of NSCLC patients with EGFR mutation.

NSCLC is characterized by a large number of M Φ in the TME.¹³ Given their diversity and plasticity, treatment approaches that induce a phenotype switch in tumor-associated M Φ can be effective against NSCLC. Although M Φ are known to be affected by EGFR-TKIs,^{15,16} it is unclear whether inducing their transformation can inhibit the progression of NSCLC with EGFR^{T790M} mutation. FMT is a nanomaterial with good biocompatibility and low toxicity that has been approved by the US Food and Drug Administration for the treatment of anemia.^{46,47} CpG has been used for cancer therapy in clinical trials.³⁰ Many studies have demonstrated the advantages of combined immunotherapy over monotherapy in cancer treatment,^{48,49} and our previous studies also showed that the combined treatment of FMT and TLR3 agonist poly (I:C) induced synergistically

induces macrophage activation for melanoma regression.²² Since they are both activators of M Φ , in this study we investigated whether FMT and CpG used in combination can suppress NSCLC by inducing M Φ activation.

IL-12 blocked the migration and invasion of lung adenocarcinoma cells⁵⁰ and suppressed lung tumor growth, thereby prolonging the survival of lung cancer-bearing mice⁵¹ while TNF- α is a cytotoxic protein produced by M Φ that can directly induce apoptosis in NSCLC cells.^{52,53} In the present study, we found that FMT/CpG was relatively non-toxic to H1975 cells, whereas FMT/CpG-pretreated M Φ supernatant inhibited H1975 cell proliferation and induced apoptosis, which would be related to the anti-tumor effects of IL-12 and TNF- α as well as other cytotoxic cytokines generated by M Φ induced by FMT/CpG. The caspase family plays critical regulatory roles in cell apoptosis.^{51,54} In our study, FMT/CpG-pretreated M Φ supernatant significantly upregulated the protein levels of apoptosis-related Cleaved Caspase-3 and its substrate Cleaved PARP in H1975 cells, indicating that the cell apoptosis of H1975 was through activating caspase-3 pathway. As abnormal activation of EGFR signaling pathway leads to sustained growth of lung cancer cells.³⁹ We also observed that p-EGFR and the downstream factors p-AKT and p-mTOR were downregulated in H1975 cells exposed to FMT/CpG-pretreated M Φ supernatant. In addition, the growth of subcutaneous

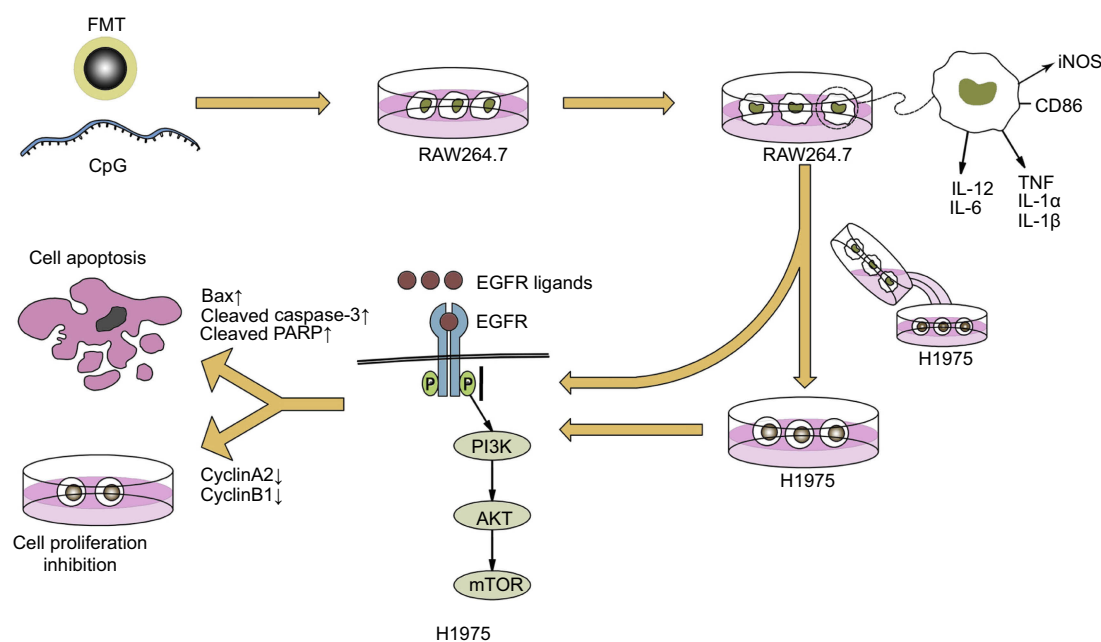


Figure 8 Schematic illustration of synergistic M Φ activation by FMT and CpG for the treatment of NSCLC with EGFR^{T790M} mutation.

Abbreviations: EGFR, epidermal growth factor receptor; FMT, ferumoxytol; M Φ , macrophages; NSCLC, non-small cell lung cancer.

xenograft tumors in mice was inhibited by treatment with FMT/CpG, which was accompanied by a decline in the EGFR-positive tumor cell fraction and increased M1 type macrophage infiltration. Thus, the combination of the two agents synergistically activated M Φ through upregulation of tumoricidal genes and induction of apoptosis, which was achieved by inhibiting the phosphorylation of EGFR and its downstream signaling in NSCLC cells (Figure 8).

Although systemic administration of TLR-9 agonists has been unsuccessful,^{55,56} local intratumoral injection of CpG ODN is still an effective strategy for tumor treatment by activation of innate responses.²⁸ Gallotta et al found that delivery of a TLR9 agonist through the airways could effectively render lung tumors permissive to PD-1 blockade by promoting optimal CD4⁺ and CD8⁺ T-cell Interplay,⁵⁷ which characterizes a strategy to apply localized TLR9 stimulation to a tumor type not accessible for direct injection. Our previous studies have showed that, compared with combined treatment with FMT/PIC, systemic administration of FP-NPs surface functionalized with poly (I:C) showed a better inhibition of lung metastasis.²² Based on the above findings, we are here to initially explore the effects of FMT combined with CpG on NSCLC with EGFR T790M mutation. Interestingly, similar to the previous results, we found that the combination of FMT and CpG significantly inhibited tumor growth in mice by synergistically activating macrophages. Although this route of administration has some limitations in clinical application it provides a good reference for us to explore novel methods for CpG delivery in the future.

Conclusion

FMT combined with CpG induced the activation of M Φ towards a tumoricidal phenotype; this was accompanied by increased apoptosis and suppression of cell proliferation and EGFR signaling in NSCLC cells. Our findings for the first time provide evidence of FMT/CpG as a novel and effective treatment for NSCLC with EGFR^{T790M} mutation.

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Disclosure

The authors report no conflicts of interest in this work.

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