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A fumigaclavine C isostere alleviates Th1-mediated experimental colitis via competing with IFN-γ for binding to IFN-γ receptor 1

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ABSTRACT

Interferon gamma (IFN-γ) signaling in T cells plays an important role in developing T helper 1 (Th1)-mediated inflammation. Selective regulation of IFN-γ signaling is an attractive strategy for treating Th1-mediated immune diseases. In this study, we aimed to explore possible means of targeting IFN-γ signaling by using small molecule compound. A synthetic small molecule FC9 was identified as it selectively inhibited IFN-γ signaling in T cells without suppressing interleukin 4 (IL-4) signaling. Furthermore, FC9 inhibited IFN-γ-induced Janus kinase 2 (JAK2) activation via competing with IFN-γ for binding to IFN-γ receptor 1 (IFN-γ R1). Interestingly, we found that FC9 bound to IFN-γ R1 and selectively suppressed Th1 but not Th2 immune response in T cells, resulting in an improvement in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. In conclusion, FC9-induced competitive blockade of IFN-γ R1 for selective inhibition of IFN-γ signaling, demonstrated a novel mean of targeting IFN-γ signaling. These findings could lead to increased options for the treatment of Crohn's disease and other Th1-mediated inflammatory diseases.

1. Introduction

Crohn's disease and ulcerative colitis are two main types of inflammatory bowel disease (IBD) [1], which are chronic relapsing inflammatory disorders mediated by $CD4^+$ T cells in a genetically susceptible host [2]. Crohn's disease is characterized by increased production of interferon gamma (IFN-γ) by T helper 1 (Th1) cells [3]. IFN-γ can induce signal transducer and activator of transcription 1 (STAT1) activation and T-box expressed in T cells (T-bet) expression in T cells via binding to IFN-γ receptor (IFN-γ R) [4]. In the absence of STAT1 in mice, experimental colitis are attenuated [5], and T-$bet^{-/-}$ T cells fail to induce colitis in adoptive transfer experiments [6]. Thus, IFN-γ signaling is critical for developing Th1-mediated colitis. Th2 cell associated cytokines IL-5 and IL-13 are associated with ulcerative colitis [1]. Th17 cell associated cytokines such as IL-17A and IL-17F, increased in IBD patients, promote intestinal inflammation [2]. Considering that, we think blocking IFN-γ signaling is an attractive treatment strategy. However, current immunosuppressants could suppress multiple STATs simultaneously. For example, dexamethasone strongly inhibits the activation of IFN-γ-induced STAT1, IL-6-induced-STAT3, IL-2-induced STAT5 and IL-4-induced STAT6 [7]. The broad targeting of multiple STATs may cause side effects, thereby limiting the clinical applications. The inhibition of STAT6 by dexamethasone may lead to an exacerbation of acute dextran sulfate sodium-induced colitis [8]. Thus, targeting IFN-γ signaling should be tightly controlled. However, selectively targeting IFN-γ signaling remains a challenge.

Fumigaclavine C (FC) produced by *Aspergillus fumigatus* (strain No. CY018) has been reported to have an anti-inflammatory activity [9]. In order to screen selective immunosuppressants, 77 isosteres of FC were synthesized. There were two steps for the screening procedure. First, compounds that suppressed T cell proliferation or T cell activation were excluded. Second, the rest compounds that suppressed IFN-γ signaling more than 50% were selected. After these two steps, several compounds were selected for further study.

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In this study, we identified a novel synthetic small molecule compound FC9 as a potential drug candidate for selective inhibition of IFN-γ/STAT1 signaling without affecting IL-4/STAT6 signaling. Furthermore, FC9 attenuated trinitrobenzene sulfonate (TNBS)-induced colitis in mice with an inhibition of IFN-γ signaling in vivo. Interestingly, FC9 bound to IFN-γ R1 on the surface of T cells and directly competed with IFN-γ for binding to IFN-γ R1, which suppressed the activation of IFN-γ-induced JAK2 and STAT1. Our findings reveal a novel mean for selective regulation of IFN-γ signaling via competing with IFN-γ for binding to IFN-γ R1 by using FC9, which may contribute to an improvement in Th1-type intestinal inflammation.

2. Materials and methods

2.1. Mice

C57BL/6 mice (6–8 weeks of age, 18–20 g) were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Mice were housed in specific-pathogen-free (SPF) facility. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and related ethical regulations of Nanjing University.

2.2. Reagents

FC9 was synthesized by our laboratory. For in vitro experiments, FC9 was dissolved at a concentration of 10 mM in DMSO as a stock solution, stored at −20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. TNBS and CsA were purchased from Sigma–Aldrich (St. Louis, MO). Recombinant murine IFN-γ, IL-4 and human IFN-γ were purchased from PeproTech (Rocky Hill, NJ). Anti-CD3, anti-CD28, and anti-IFN-γ R1 antibodies were purchased from BD PharMingen (San Diego, CA). Antibodies against pY-STAT1, STAT1, pY-STAT6, STAT6, pY-JAK2 and JAK2 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against GAPDH and IFN-γ R were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies used in flow cytometry were purchased from eBioscience (San Diego, CA).

2.3. Cell culture and T cell isolation

T cells derived from mice were incubated in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% NBS under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. $CD4^+$ T cells and naive T cells from spleen and lymph node cells were isolated using $CD4^+$ T Cell isolation kit and $CD4^+$ $CD62^+$ naive T cell isolation kit from Miltenyi Biotec (Auburn, CA) according to the manufacturer's instructions.

2.4. Th1, Th2 and Th17 cell differentiation in vitro

Naive T cells were purified from mice with MicroBeads and differentiated in vitro. For Th1, Th2 and Th17 differentiation, naive T cells were incubated with plate-bound mAbs of anti-CD3 and anti-CD28 in the presence of Th1 condition (10 ng/ml IL-12, 5 μg/ml anti-IL-4 mAb), Th2 condition (10 ng/ml IL-4, 5 μg/ml anti-IFN-γ mAb, 5 μg/ml anti-IL-12 mAb) or Th17 condition (20 ng/ml IL-6, 1 ng/ml TGF-β, 5 μg/ml anti-IFN-γ mAb, 5 μg/ml anti-IL-4 mAb) for 5 days. Differentiated Th cells were washed and re-stimulated with plate-bound anti-CD3 mAb for 24 h, and cell supernatants were used for measuring cytokine levels by ELISA (R&D Systems, Minneapolis, MN).

2.5. Cytokine analysis by ELISA

Cytokine levels of IFN-γ and IL-5 in culture supernatants or serum were measured using ELISA kits from R&D systems (Minneapolis, MN).

2.6. Flow cytometry

For intracellular staining of IFN-γ, lymph node-derived T cells were activated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml, Sigma–Aldrich, St. Louis, MO), ionomycin (1 μg/ml, Sigma–Aldrich, St. Louis, MO) and monensin (1 μg/ml, eBioscience, San Diego, CA) for 4 h. Cultured cells were stained with PeCy5-anti-CD3, fixed, permeabilized, and then stained with PE-anti-IFN-γ. For detection of surface IFN-γ R expression, T cells were stained with anti-IFN-γ R1, and then with PE-anti-IgG. Flow cytometric analysis was performed on BD FACS Calibur (BD Biosciences) and results were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.7. Induction of TNBS-induced colitis in mice and drug administration

Murine colitis was induced by TNBS following a published protocol [10] with some modifications. Briefly, adult male C57BL/6 mice were sensitized with 150 μl of 1% TNBS solution on abdominal skin 7 days before challenge. On the day of challenge, mice were lightly anaesthetized and TNBS solution (2.5 mg TNBS in 50% ethanol solution) was administrated intrarectally. In the sham control, mice received 50% ethanol alone. Animals were monitored daily for the loss of body weight and survival. On day 0, 1, and 2, FC9 (5, 10, 20 mg/kg/d, dissolved in water, pH = 4) was administered intragastrically (i.g.). CsA (10 mg/kg/d, dissolved in olive oil) was intraperitoneally (i.p.) administered each day. Mice in vehicle-treated group received administration of water (pH = 4, i.g.).

2.8. Macroscopic and histologic grading of colitis

Colons and paraffin sections were examined and graded by macroscopic and microscopic analysis following previously reported methods [7]. Briefly, nine parameters were taken into account to score colonic damage macroscopically: erythema (0, 1 on less than 1 cm, 2 on more than 1 cm), hemorrhage, edema, stricture formation, ulceration, and fecal blood, presence of mucus, diarrhea, and adhesions (0, 1 mild, 2 severe). Each parameter was awarded 1 point except erythema and adhesions. Grading was performed in a blinded fashion. Histological evaluation of H&E-stained colonic sections was graded from 0 to 4 as follows in a blinded fashion: 0, no signs of inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss, and focal loss of crypts; 4, transmural infiltrations, massive loss of goblet cell, extensive fibrosis, and diffuse loss of crypts.

2.9. Immunohistochemical studies

Frozen samples were cut in 8-μm sections on a CM1950 cryostat (Leica, Wetzlar, Germany) and mounted on positively charged glass slides (CITOGLAS, Jiangsu, China). Sections were fixed in 4% paraformaldehyde for 15 min and subsequently brought to water in PBS. H&E staining was performed according to standard procedures. Frozen sections for immunohistofluorescence were permeabilized in 0.3% Triton X-100 (Sunshine Biotechnology Co., Ltd., Nanjing, China) at room temperature for 20 min, followed by incubation with primary antibodies at 4 °C for 8 h. Anti-pY-STAT1 (1:50) was used to detect p-STAT1. After three washes in PBS, fro-

zen sections were incubated at 37 °C for 1 h with the secondary antibody: goat anti-rabbit IgG H&L (Alexa Fluor® 594) (Thermo Fisher Scientific). Frozen sections were stained with DAPI (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). They were examined with laser-scanning microscopy (Nikon C2, Japan).

2.10. Real-time RT-PCR analysis

RNA was extracted from cells (5×10^6) or frozen colonic tissues (20 mg) using Trizol Reagent (Invitrogen). One microgram of RNA was reverse transcribed to cDNA. The primer sequences used in PCR were as follows: GAPDH, 5′-AACGACCCCTTCATTGAC and 3′-CACGACTCATACAGCACCT; T-bet, 5′-CTCAGGTGGCTGGCTTTC and 3′-ATTCGTTCCTGCCGCTTA; GATA-binding protein 3 (GATA3), 5′-GGGTTCGGATGTAAGTCG and 3′-GTAGGGACTCGGTGTAGA; retinoic acid-related orphan receptor variant 2 (RORC2), 5′-GACCCACACCTCACAAATTGA and 3′-AGTAGGCCACATTACACTGCT; IL-12p35 5′-CTGTGCCTTGGTAGCATCTATG and 3′-GCAGAGTCTCGC CATTATGATTC; IL-27 5′-CTGTTGCTGCTACCCTTGCTT and 3′-CACTC CTGGCAATCGAGATTC. Reactions were run in triplicate using GAPDH as the internal RNA control on an ABI 7000 Thermocycler (Applied Biosystems, Framingham, MA).

2.11. Western blot

Proteins were extracted in lysis buffer, separated by SDS–PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). The membranes were blocked with 5% nonfat milk for 1 h at room temperature, probed with specific primary antibodies overnight at 4 °C, and then incubated with a HRP-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.12. Electrophoretic mobility shift assay (EMSA)

Nucleic protein from T cells was extracted and subjected to EMSA. The double-stranded oligonucleotide probe containing GAS (5′ biotin-AGCCTGATTTCCCCGAAATGACGGC-3′ and 5′ biotin-GCCGTCATTTCGGGGAAATCAGGCT-3′) corresponding to the STAT1 consensus DNA-binding site was obtained from Invitrogen (Carlsbad, CA). Reactions for nuclear protein-DNA binding were performed using LightShift chemiluminescent EMSA kit from Pierce (Rockford, IL). Specificity of DNA–protein complex was confirmed by competition with a 100-fold excess of unlabeled GAS binding sequences (Invitrogen, Carlsbad, CA).

2.13. Atomic force microscopy (AFM) tip preparation

AFM silicon nitride (Si_3N_4) tips (type NP with a radius of 20–60 nm, from Veeco, Santa Barbara, CA) were first incubated with chromic acid for 20 min to generate Si-OH groups. Then they were transferred to a solution of 1.0% (v/v) MPTMS in toluene, incubated for 2 h at room temperature, and rinsed thoroughly with toluene to be modified with –SH groups. After being dried with N_2, the tips were activated by incubation in 1 mg/ml NHS-PEG-MAL, the cross-linker, in DMSO for 3 h at room temperature and then rinsed thoroughly with DMSO to remove any unbound NHS-PEG-MAL. The NHS-PEG-MAL was conjugated to the –SH groups on the AFM tips via its MAL end. These activated tips were immersed into a protein (human IFN-γ) solution (5 ng/ml in PBS) and incubated at room temperature for 0.5 h. The proteins were bound via their intrinsic amine groups to the NHS end of the PEG derivative. After rinsing with PBS, the protein-modified tips were stored in PBS at 4 °C until use.

2.14. AFM force measurements

HeLa cells which expressed surface IFN-γ R were treated with FC9 (10 μM) or anti-IFN-γ R (1 μg/ml) for 12 h. Then the force measurements of the IFN-γ-modified AFM tip on the living HeLa cells were carried out on a PicoSPM II with PicoScan 3000 controller and a large scanner (Molecular Imaging, Tempe, AZ). The AFM scanner was mounted on an inverted fluorescence microscopy (Olympus IX71, Japan). The loading rate of force measurements was 1.0×10^4 pN/s. The force curves measured in living HeLa cells were recorded and analyzed by PicoScan 5 software (Molecular Imaging, Tempe, AZ). All forces were measured with contact mode at room temperature.

2.15. Computer docking

To prepare the ligand, the structure of FC9 was drawn by ChemDraw software (http://www.acdlabs.com). The dimensional structure of human IFN-γ R (PDB code 1FG9) was obtained from the RCSB Protein Data Bank. Autodock 4.2 [11] and Auto DockTools 1.5.4 (ADT; http://mgltools.scripps.edu/) were used for docking of FC9 on human IFN-γ R. A cubic grid of 95, 222 and 144 points in X-, Y- and Z-directions was built, grid centered on 4.2537 point (X direction), 0.0753 point (Y direction) and −21.9794 point (*Z* direction) of the receptor protein. In order to calculate the energy map, a grid spacing of 0.375 Å was used in our docking protocol. In the present study, docking was done with Lamarckian genetic algorithm with population of 200, which was used for calculation of the docking conformations. The maximum number of energy evaluations was set to 2,500,000.

2.16. Isothermal titration calorimetry

An isothermal titration calorimeter (VP-ITC, Microcal Inc., Northampton, MA) was used to measure enthalpy values. After setting the temperature of solutions at 20 °C in the degassing device, the apparatus was equilibrated at 22 °C for at least half an hour before the measurement. The FC9 or FC96 solution was titrated with 50 successive 5 μL injections of IFN-γ R1 solution. Each addition lasted 12 s with an interval of 800 s between consecutive injections. The solution in the reaction cell was stirred at 310 rpm throughout the experiments. The heats of dilution from the blank titration of the IFN-γ R1 into the buffer solution and the buffer into FC9 or FC96 solution were subtracted from the raw data. Thermodynamic parameters, including binding constant (K), enthalpy (ΔH), entropy (ΔS), and binding stoichiometry (N) (molar ratio monomeric IFN-γ R1 /FC9 or FC96), were calculated by iterative curve fitting of the binding isotherms. The "2-binding-site" model from the software Microcal ORIGIN provided with the ITC apparatus was used. The Gibbs free energy was calculated from the equation $\Delta G = \Delta H - T\Delta S$.

2.17. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistically evaluated by Student's *t* test when only two value sets were compared, and one-way ANOVA followed by Dunnett's test when the data involved three or more groups. $P < 0.05$ was considered to be significant.

3. Results

3.1. FC9 selectively inhibited IFN-γ/STAT1 signaling pathway in T cells

FC9, a novel small molecule derived from Fumigaclavine C, was selected from a screening for selective immunosuppressive

chemicals (Fig. 1A). Interestingly, phosphorylation, but not expression, of STAT1 was markedly inhibited by FC9 in a dose- (Fig. 1B) and time-dependent (Fig. 1D) manner, while 1 μM Cyclosporine A (CsA) inhibited the activation of both IFN-γ-induced STAT1 and IL-4-induced STAT6 (Fig. 1B and C). FC9 did not inhibit the activation of IL-4-induced STAT6 (Fig. 1C and E), IL-6-induced STAT3 (Fig. 1F) or IL-2-induced STAT5 (Fig. 1G). Consistent with its effects on STAT1 activation, FC9 reduced the binding of STAT1 dimer to a gamma-activated sequence (GAS) element in $CD4^+$ T cells treated with IFN-γ (Fig. 1H).

3.2. FC9 inhibits the expression of the IFN-γ-induced Th1 transcription factor, T-bet, Th1 differentiation and the production of Th1 cytokine, IFN-γ, in polarized T cells

IFN-γ can induce the expression of T-bet, a transcription factor that directs Th1 lineage commitment [12], and IL-4 can induce the early expression of GATA3, a transcription factor that is crucial to Th2 cytokine production [13]. FC9 inhibited the expression of T-bet induced by IFN-γ in T cells (Fig. 2A) in a dose-dependent manner without affecting the expression of GATA3 induced by IL-4 (Fig. 2B). As IFN-γ is critical for Th1 cell differentiation, we investigated effects of FC9 on Th1 cell differentiation. FC9 inhibited Th1 but not Th2 or Th17 cell differentiation and decreased T-bet but not GATA3 or RORC2 mRNA level (Fig. 2C and D). Furthermore, IFN-γ production in Th1-polarized T cells but not IL-5 production in Th2-polarized T cells was inhibited by FC9 (Fig. 2E and F).

3.3. FC9 alleviates TNBS-induced experimental colitis with an inhibition of IFN-γ/STAT1 signaling

Mice with TNBS-induced colitis have a Th1-mediated gut inflammation characterized by greatly increased production of IFN-γ [14,15]. The specific effect of FC9 on STAT1 signaling was further examined for its potential to alleviate TNBS-induced colitis in mice. A significant body weight loss, an increase in the macroscopic damage scores and colon wall thickness were observed in the mice with colitis. In contrast, a significant attenuation of these responses was found in the colons of mice treated with either 10 or 20 mg/kg of FC9 or with 10 mg/kg CsA (Fig. 3A and B). In addition, the infiltration of inflammatory cells in the colon of mice with TNBS-induced colitis was inhibited by FC9 (Fig. 3C). Confocal

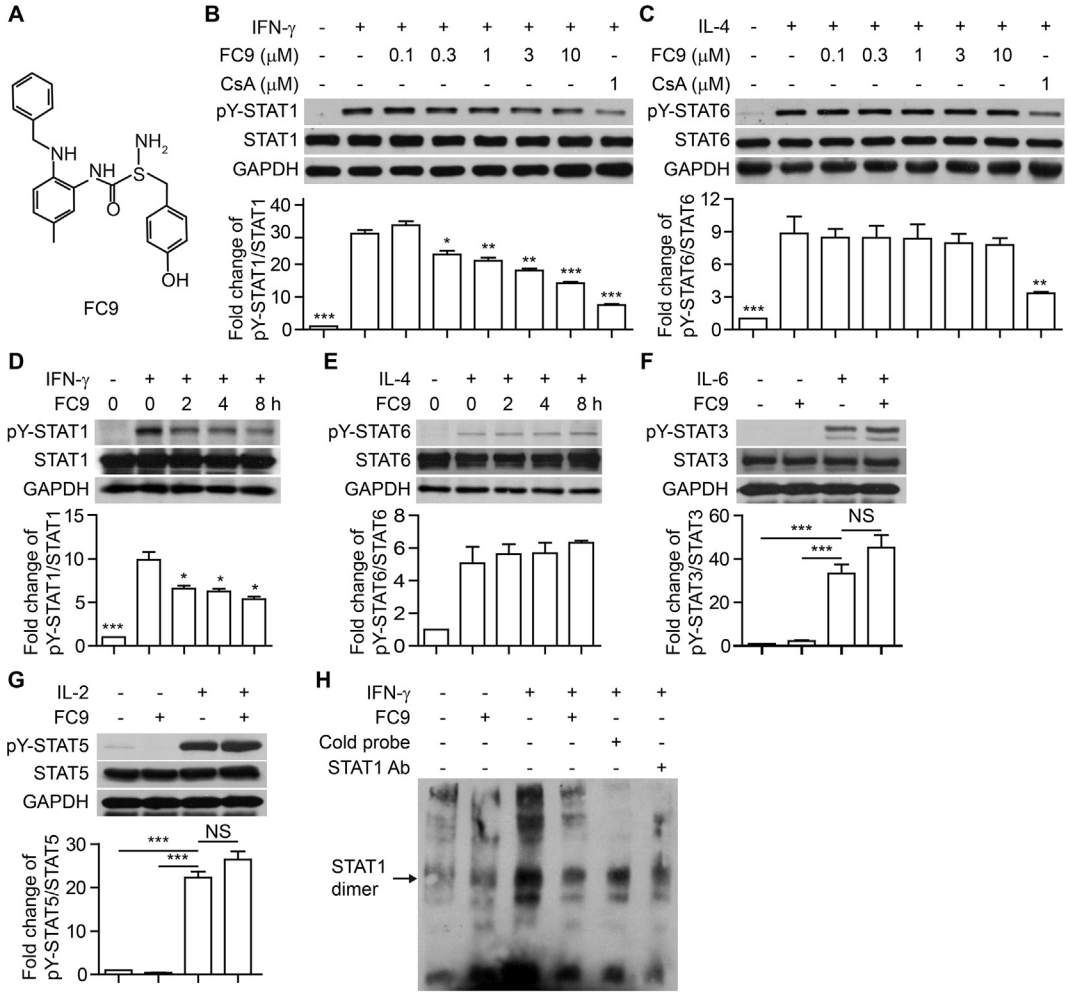

Fig. 1. Selective inhibition of IFN-γ/STAT1 signaling pathway by FC9 in T cells. (A) Structure of FC9. (B-D) Naïve T cells were treated with FC9 or CsA for indicated time at 10 μM (B) or 8 h (D), following IFN-γ (10 ng/ml) stimulation for 30 min. Naïve $CD4^+$ T cells were treated with FC9 or CsA for indicated time at 10 μM (C) or 8 h (E), following IL-4 (10 ng/ml) stimulation for 30 min. Data are representative of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with stimulator-treated group. (F) Naïve T cells were treated with FC9 (10 μM) for 8 h following IL-6 (25 ng/ml) stimulation for 30 min. (G) Naïve $CD4^+$ T cells were treated with FC9 (10 μM) for 8 h following IL-2 (10 ng/ml) stimulation for 30 min. (B-G) After these incubations, the proteins were extracted and subjected to Western blot analysis. (H) Naïve $CD4^+$ T cells were treated with FC9 for 8 h following IFN-γ (10 ng/ml) stimulation for 30 min. The nuclear proteins were isolated and subjected to EMSA analysis. All data shown here are representative of three independent experiments. Quantification of western blot was done with ImageJ (mean ± SEM; n = 3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant. Data are representative of at least three independent experiments.

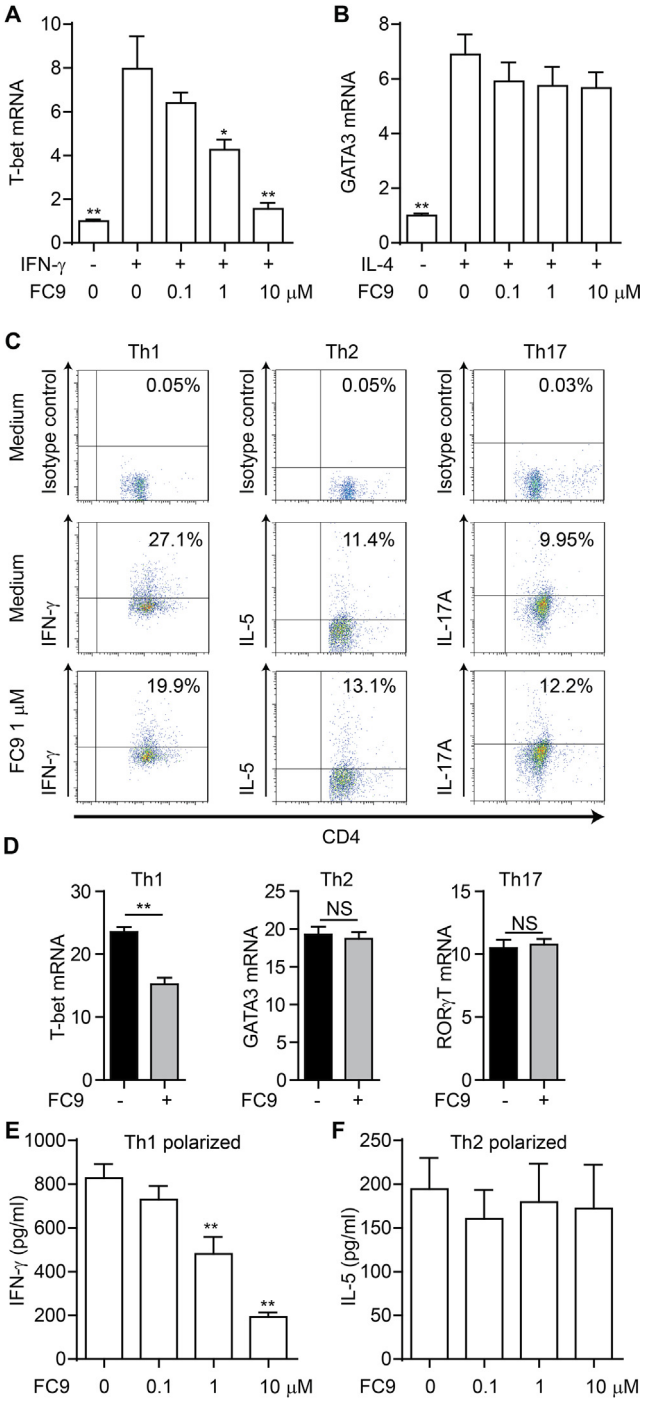

Fig. 2. Selective inhibition of T-bet expression, Th1 differentiation, and Th1 cytokine production by FC9. (A-B) Real-time PCR analysis of T-bet and GATA3 expressions in T cells cultured with or without FC9 for 8 h and then treated with murine IFN-γ (25 ng/ml) (A) or IL-4 (10 ng/ml) (B) for 6 h. One-way ANOVA revealed a significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$) compared with the only stimulator-treated group. (C) Naïve T cells were activated by anti-CD3 mAb 1 μg/ml plus anti-CD28 mAb 0.5 μg/ml and cultured with Th1 differentiation condition (IL-12 at 10 ng/ml plus anti-IL-4 mAb at 10 μg/ml), Th2 differentiation condition (IL-4 at 10 ng /ml plus anti-IL-12 and anti- IFN-γ antibody) and Th17 differentiation condition (IL-6 at 10 ng/ml, TGF-β at 1 ng/ml plus anti-IL-4 and anti- IFN-γ antibody) with or without FC9 (1 μM) for 5 days. Then cells were assessed by FACS. (D) The mRNA expression of Th1, Th2 and Th17 transcription factors were determined by real-time PCR. (E-F) Polarized Th1 and Th2 cells were treated or not with FC9 for 72 h, and culture supernatants were subjected to ELISA. The values represent the mean ± SEM from three independent experiments. $^{**}P < 0.01$.

imaging revealed that an increase of pY-STAT1 (red) expression was observed in the colon of mice with TNBS-induced colitis (Fig. 3D). The increase of pY-STAT1 expression was inhibited by FC9 (Fig. 3D). Consistently, FC9 inhibited T-bet mRNA expression in the colonic tissue in a dose-dependent manner (Fig. 3E). In addition, FC9 reduced the mRNA expression of IL-12 but not IL-27 in the colon of mice treated with TNBS (Fig. 3E). Thus, FC9 might affect other cells such as myeloid cells via regulating IFN-γ signaling in vivo.

3.4. FC9 competitively inhibits IFN-γ binding to IFN-γ R1 for controlling IFN-γ-induced STAT1 activation

To determine how FC9 specifically inhibits STAT1 activation, we first performed IFN-γ and IFN-γ R binding force measurements by atomic force microscopy. The atomic force microscopy tip was modified with IFN-γ and moved to the HeLa cells which expressed IFN-γ R. The rupture forces were detected when the tip and cell were brought into and out of contact. Cells without FC9 treatment showed a strong binding force between IFN-γ and IFN-γ R1, while FC9-treated cells had a weak binding affinity (Fig. 4A and B).

Further, we examined the effect of FC9 on the activation of JAK2, an upstream kinase that induces STAT1 phosphorylation. FC9 inhibited the phosphorylation of JAK2 in T cells stimulated with IFN-γ (Fig. 4C). In addition, a higher dose of IFN-γ (100 ng/ml) could reverse the inhibition of STAT1 activation by FC9 (Fig. 4D). These data suggest that FC9 competitively inhibited the binding of IFN-γ to IFN-γ R1.

Various factors regulate IFN-γ/STAT1 signaling pathway. FC9 may also affect other factors besides competing IFN-γ binding to IFN-γ R. We analyzed effects of FC9 on protein synthesis and degradation by using protein proteasome inhibitor MG132 and synthesis inhibitor cycloheximide (CHX). The reduced level of phosphorylated STAT1 observed in FC9-treated T cells was not altered by the treatment of MG132 or CHX (Fig. 4E and F), suggesting that the effect of FC9 did not depend on protein synthesis and degradation. We examined the level of suppressor of cytokine signaling (SOCS) family in this process. SOCS proteins are important negative regulators in cytokine-triggered signaling pathways and also play an important part in regulating STAT1 signaling [16,17]. When stimulated with IFN-γ, T cells expressed augmented amount of SOCS1 as well as SOCS3. Treatment of FC9 did not affect the expression of SOCS1 or SOCS3 induced by IFN-γ (Data not shown), indicating that the effect of FC9 did not depend on SOCS1 or SOCS3. We also examined effects of FC9 on phosphatase activity. Sodium orthovanadate is a commonly used phosphatase inhibitor [18]. Co-culture of sodium orthovanadate did not affect the inhibitory effect of FC9 on STAT1 phosphorylation (Fig. 4G). In addition, FC9 did not affect the dephosphorylation process of pY-STAT1 when the IFN-γ stimulation was withdrawn (Fig. 4H). In addition, FC9 did not inhibit the anti-IFN-γ R1 antibody (clone 2e2) binding to IFN-γ R1 (data not shown). Thus, these data supported that FC9 inhibited IFN-γ signaling via competing with IFN-γ for binding to IFN-γ R1.

3.5. FC9 binds to IFN-γ R1

Next we performed computer docking to determine if FC9 could bind to IFN-γ R1. The binding energy value, intermolecular energy value, internal energy value, torsional energy value and unbound extended energy value in binding between FC9 and IFN-γ R1 from Autodock 4.2 were found to be −10.37, −12.4, −1.3, 2.68 and −0.64, respectively. In addition, Ki value was 24.94 nM and ref rms value was 17.05. These data indicated FC9 might bind to IFN-γ R1. Furthermore, we found that FC9 could form hydrogen bonds with Phe134 (1.734 Å) and Glu180 (1.323 Å) of human IFN-γ R (Fig. 5A). Notably, the binding pocket of FC9 in human IFN-γ R (Fig. 5B and C, red circles) was also the binding site of

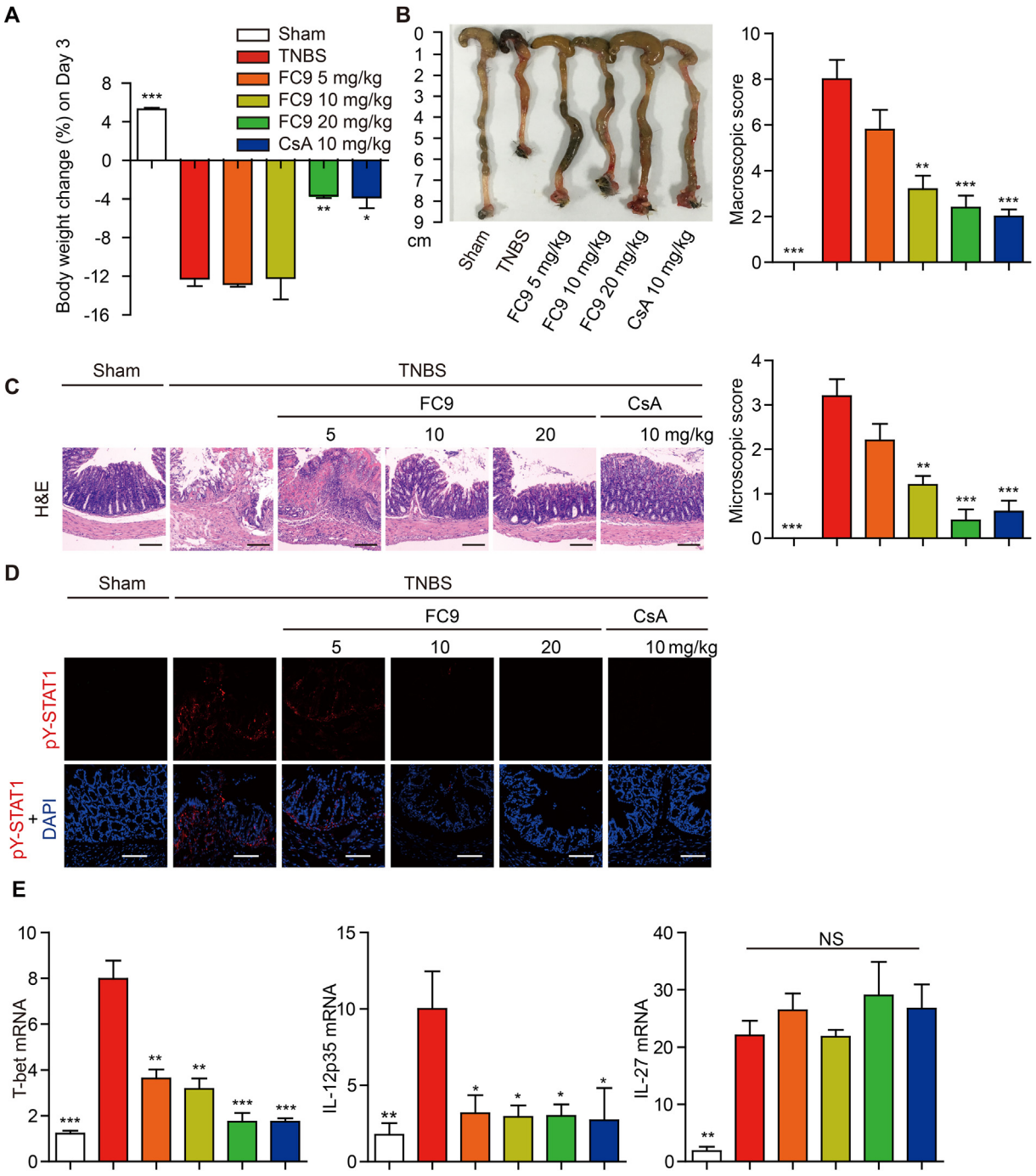

Fig. 3. FC9 competitively inhibits IFN-γ binding to IFN-γ R1 for controlling IFN-γ-induced STAT1 activation (A) Percentage of body weight changes. (B) Macroscopic damage scores. (C) Histopathologic analyses were performed on hematoxylin- and eosin-stained sections of colons (scale bar, 100 μm) and microscopic damage scores (D) Confocal imaging of pY-STAT1 (red) and DAPI (blue) in the colonic tissue (scale bar, 200 μm). (E) Real-time PCR analysis of T-bet, IL-12 and Il-27 mRNA expression in the colon tissues. A significant difference was revealed following one-way ANOVA analysis ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with TNBS-treated mice). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IFN-γ to human IFN-γ R (Fig. 5C, blue circles). These data suggest that FC9 might be a competitor of IFN-γ for IFN-γ signal transduction. Furthermore the binding between FC9 and IFN-γ R1 was demonstrated by isothermal titration calorimetry (Fig. 5D). FC96 was used as a negative control (Fig. 5E).

4. Discussion

Targeting JAK-STAT signaling pathway is an attractive strategy for treating immune-mediated diseases [19]. Here, we found a novel mean of selectively targeting IFN-γ signaling by using a small molecule FC9. FC9 was selected from a screening of selective immunosuppressive chemicals. FC9 did not suppress proliferation of T cells at 10 μM nor T cell activation. In contrast, CsA suppresses T cell activation, T cell proliferation and Th1 response. Thus, FC9 may show fewer side effects due to the higher specificity.

Both Th1- and Th2-mediated immunity is tightly regulated as excessive activation may cause various immune disease [20–23]. Remaining Th1/Th2 balance is important for treating autoimmune disease [3,24]. Th1/Th2 balance is not only controlled by Th1 cells and Th2 cells, but also by various regulatory factors [25]. In

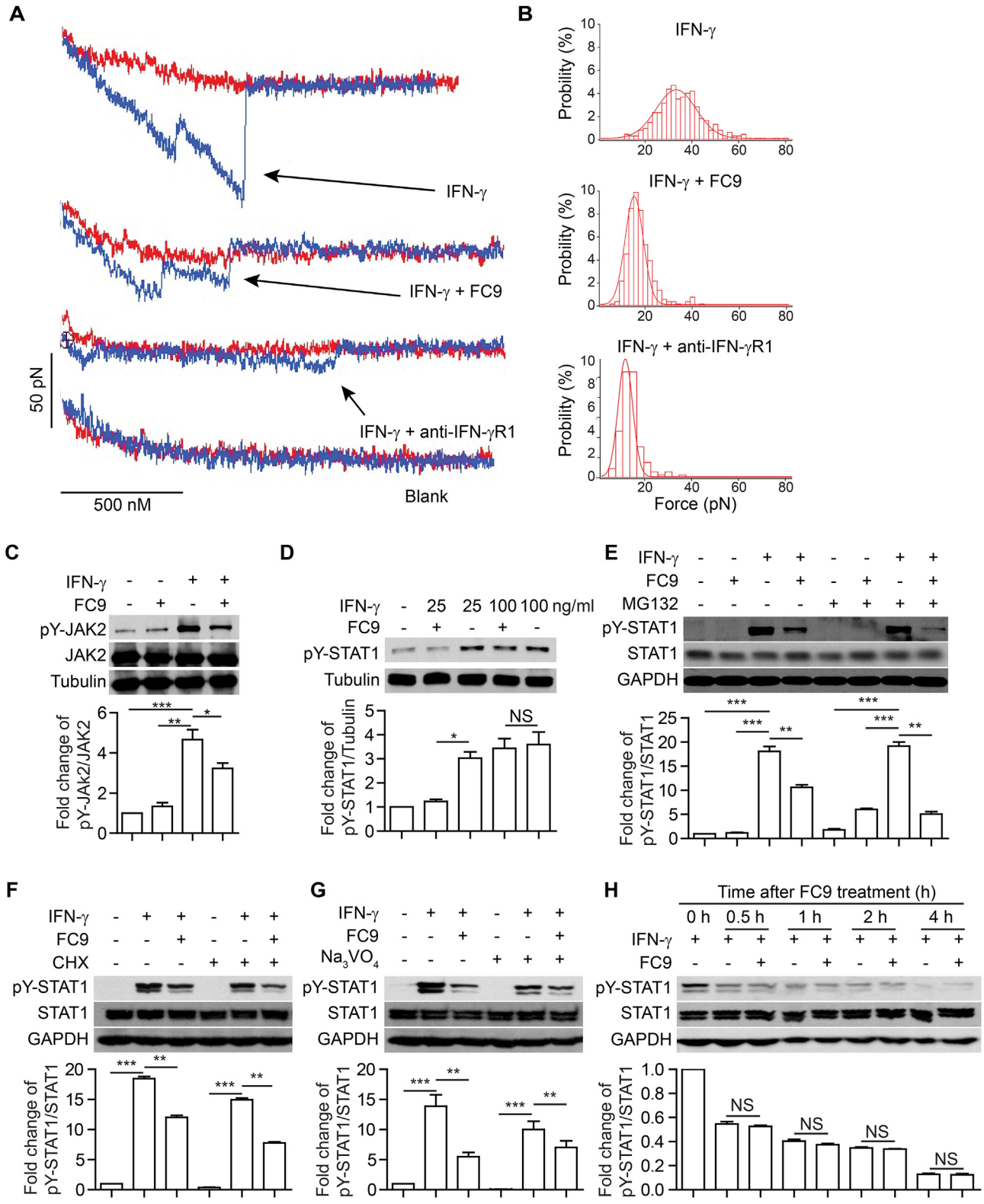

Fig. 4. Competitive blockade of interaction between IFN-γ and IFN-γ R1 by FC9 Effects of FC9 on regulation factors of IFN-γ/STAT1 signaling. (A-B) HeLa cells were treated with FC9 (10 μM) or neutralization antibody of IFN-γ R1 (1 μg/ml) for 12 h. Blank or IFN-γ-modified AFM tips were used on Hela cells to assess the binding of IFN-γ/IFN-γ R. (A) Representative binding force curves. (B) Histograms of binding forces of IFN-γ/IFN-γ R. Bars represent experimental data. Solid lines represent theoretical Gaussian distribution curves. (C) Naïve T cells were incubated with FC9 (10 μM) for 8 h following IFN-γ (25 ng/ml) stimulation for 30 min. Expressions of pY-JAK2 and JAK2 were examined by western blot. (D) Naïve T cells were incubated with FC9 (10 μM) for 8 h following IFN-γ (25 ng/ml) or IFN-γ (100 ng/ml) stimulation for 30 min. (E-G) Naïve T cells were incubated with FC9 (10 μM) and MG132 (20 μM) (E), CHX (1 μg/ml) (F) or sodium orthovanadate (1 mM) (G) for 8 h, followed by stimulation of IFN-γ (25 ng/ml) for 30 min. Phosphorylation of JAK2 and STAT1 was examined by western blot. (H) $CD4^+$ T cells were stimulated with IFN-γ (25 ng/ml) for 30 min. Then the medium was replaced with RPMI 1640 without IFN-γ. IFN-γ-primed cells were treated in the presence or absence of FC9 (10 μM) for indicated time. Level of p-STAT1 was examined by western blot. All data shown here are representative of three independent experiments. Quantification of western blot was done with ImageJ (mean ± SEM; n = 3). $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant. Data are representative of at least three independent experiments.

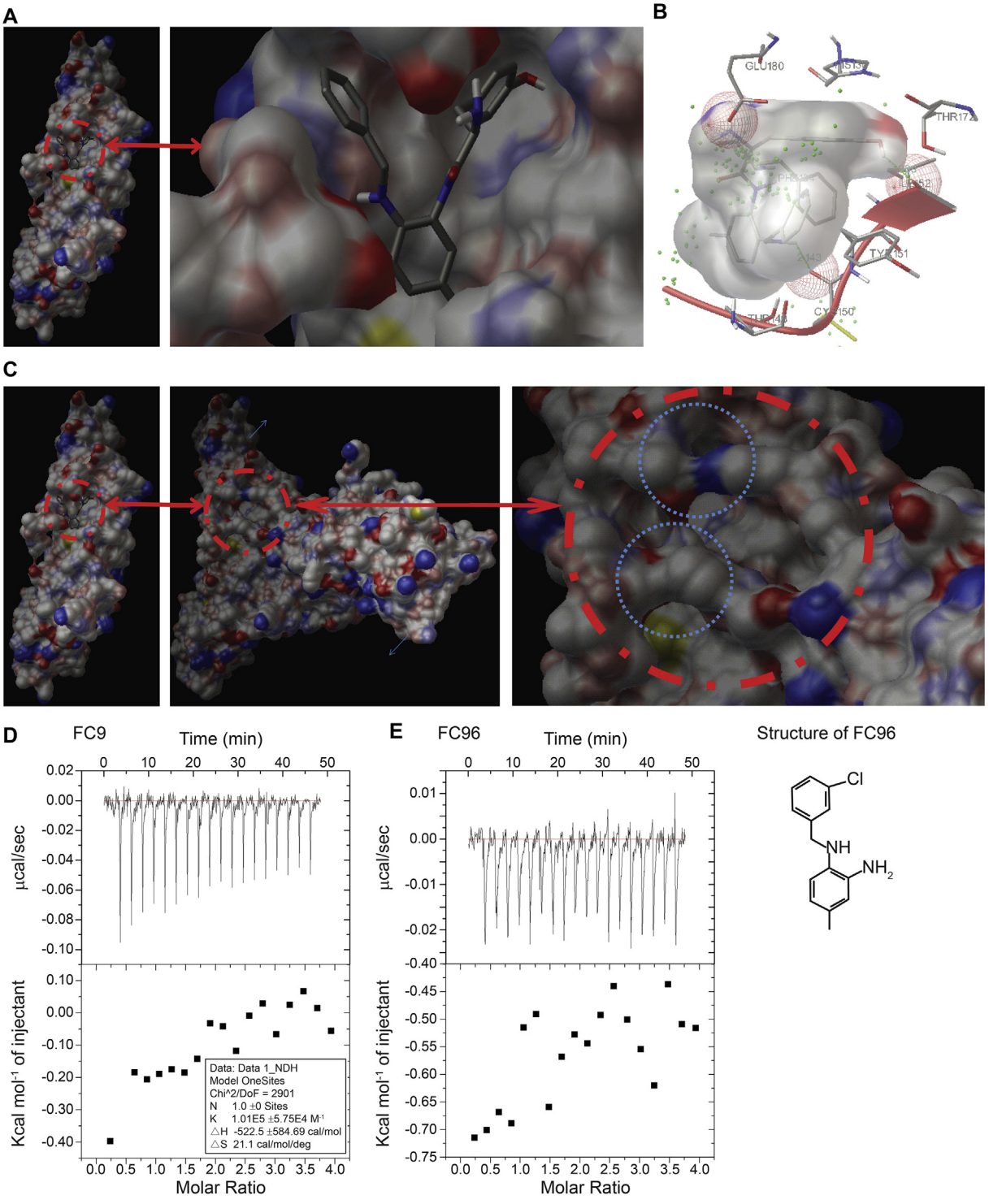

Fig. 5. Binding between FC9 and IFN-γ R1. (A-D) Computer docking of FC9 to human IFN-γ R1 was performed using Autodock 4.2 and Auto Dock Tools 1.5.4. (A) The docking result of FC9 to IFN-γ R. The red circle indicates the binding pocket of FC9. (B) The hydrogen bonds between FC9 and IFN-γ R1 at Phe134 and Glu180. (C) The binding between IFN-γ R and IFN-γ or FC9. Red circles indicate the binding pocket of FC9. Blue circles indicate the binding pocket of IFN-γ. (D) Isothermal titration calorimetry analysis of binding between extracellular domain of IFN-γ R1 and FC9. (E) FC96 was used as a control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Th1-mediated colitis, Th2 and Treg cells are anti-inflammatory factors and play protective roles [26,27]. FC9 did not inhibit activation of IL-2-induced STAT5 that promoted generation of iTreg cells [28]. FC9 inhibited Th1 immune response but not Th2 immune response. Selectively targeting Th1 cells by FC9 is more beneficial for restoring Th1/Th2 balance than CsA that inhibited both Th1 and Th2 immune responses. In addition, Th17 cells have been identified to play important roles in promoting several autoimmune diseases including IBD, maintaining mucosal barriers and contributing to pathogen clearance [1,2]. FC9 did not inhibit Th17 differentiation (Fig. 2C). As is shown in our previous work [9], Fumigaclavine C alleviated TNBS-induced experimental colitis in

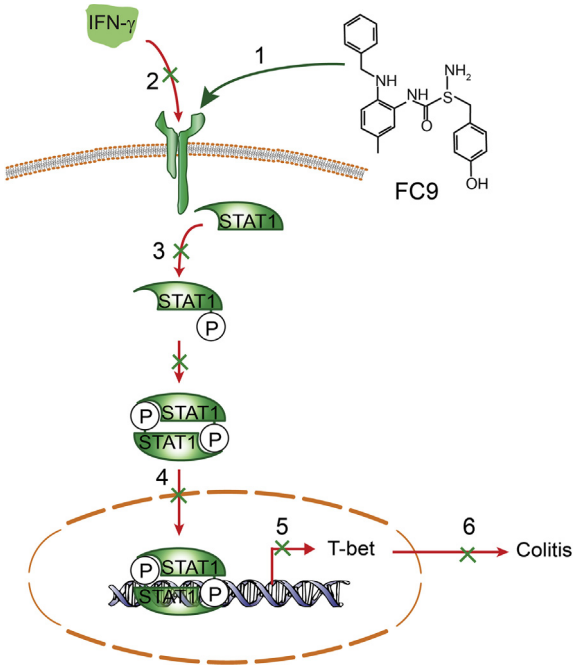

Fig. 6. A novel mode of targeting IFN-γ signaling by FC9-driven competition of IFN-γ binding to IFN-γ R. (1) FC9 bound to IFN-γ R1 directly. (2) FC9 competed with IFN-γ binding to IFN-γ R1. (3) FC9 inhibited STAT1 phosphorylation. (4) STAT1 nuclear translocation and binding to GAS were also inhibited by FC9. (5) FC9 inhibited T-bet expression. (6) TNBS-induced colitis was ameliorated by FC9.

mice. Fumigaclavine C also inhibited the expression of several proinflammatory cytokines both in vivo and in vitro, including the production of IFN-γ from lymphocytes. Here, we found that FC9 can also ameliorate TNBS-induced experimental colitis in mice and the improvement was stronger than that of Fumigaclavine C (Fig. 3). Moreover, FC9 bound to IFN-γ R1 and selectively suppressed Th1 response. Inflammatory bowel disease is a chronic inflammatory disease of the gastrointestinal tract that can present in two different forms: Crohn's disease and ulcerative colitis [29]. The TNBS-induced experimental colitis, a widely used chemically induced model of intestinal inflammation, is considered as a T cell mediated inflammatory response [30]. In the present study, we demonstrated that FC9 at 10 or 20 mg/kg, improved TNBS-induced colitis (Fig. 3) with inhibiting STAT1 activation via competing with IFN-γ for binding to IFN-γ R1 (Fig. 4), which suggested FC9 as a novel inhibitor of IFN-γ signaling. In contrast, fludarabine, another selective inhibitor of STAT1 [31], has the side effect of inhibition of DNA synthesis, which restricted its application [32]. In addition, although a phase II clinical trial of Fontolizumab has been terminated; significant decreases in C-reactive protein levels were observed [33]. We think approaches to inhibiting IFN-γ signaling remain to be improved.

In summary, the unique molecule FC9 bound to IFN-γ R1 and competitively inhibited IFN-γ binding to IFN-γ R, which induced a selective inhibition of Th1 immune response and contributed to the improvement of experimental colitis (Fig. 6). This study realized a novel mean of targeting IFN-γ signaling and provided a novel therapeutic approach in Crohn's disease and other Th1-type inflammatory diseases. This specificity has advantages over current therapies because of its potential to reduce side effects.

Conflict of interest

The authors have no conflict of interest to declare.

Author Contributions

YT carried out experiments, analyzed data and participated in writing the manuscript. JS, WG, FG, FS, TT, BZ, YC and YG carried out some experiments and provided regents. YS, XW and QX designed experiments and wrote the manuscript.

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