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A human microsatellite DNA-mimicking oligodeoxynucleotide with CCT repeats negatively regulates TLR7/9-mediated innate immune responses via selected TLR pathways

Ran Sun [a,d], Luguo Sun [b], Musheng Bao [c], Yongsheng Zhang [b], Li Wang [a], Xiuli Wu [b], Dali Hu [a], Yongjun Liu [c], Yongli Yu [a,*], Liying Wang [b,*]

[a] *Department of Immunology, Norman Bethune College of Medical Sciences, Jilin University, Changchun, 130021, China*
[b] *Department of Molecular Biology, Norman Bethune College of Medical Sciences, Jilin University, Changchun, 130021, China*
[c] *Department of Immunology and Center for Cancer Immunology Research, University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, USA*
[d] *Tissue Bank of China-Japan Union Hospital, Jilin University, Changchun, 130021, China*

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TLR activation-associated diseases;
pDC activation

Abstract A human microsatellite DNA-mimicking ODN (MS ODN) composed of CCT repeats, designated as SAT05f, has been studied for its capacity of negatively regulating innate immunity induced by TLR7/TLR9 agonists in vitro and in mice. The result showed that SAT05f could down-regulate TLR7/9-dependent IFN-α production in cultured human PBMC stimulated by inactivated Flu virus PR8 or HSV-1 or CpG ODN or imiquimod, protect D-GalN-treated mice from lethal shock induced by TLR9 agonist, not by TLR3/4 agonist. In addition, SAT05f significantly inhibit IFN-α production from purified human plasmacytoid cells (pDCs) stimulated by CpG ODN. Interestingly, SAT05f could up-regulate CD80, CD86, and HLA-DR on the pDCs in vitro, implying that SAT05f-mediated inhibition on IFN-α production could be related to the activation of pDCs. The data suggest that SAT05f could be developed as a candidate medicament for the treatment of TLR7/9 activation-associated diseases by inhibiting TLR7/9 signaling pathways.

Introduction

Viral DNA or bacterial DNA is an exogenous danger signal for immune system in mammalian. In the long time evolution of resisting virus and bacteria, the mammalian has developed pattern recognition receptors (PRR) for sensing conserved pathogen-associated molecular patterns (PAMPs), such as Toll-like receptors (TLRs). TLR3, TLR7/8, and TLR9 sense viral or bacterial nucleic acids and thereafter initiate innate immune responses [1]. Viral single-stranded RNA (ssRNA) induces large amount of IFN-α by activating TLR7/8 [2], and bacterial double-stranded DNA (dsDNA) activates TLR9 to induce inflammatory cytokines including IFN-α, TNF-α, and IL-12

* Corresponding authors. Fax: +86 431 85647872.
E-mail addresses: ylyu@mail.jlu.edu.cn (Y. Yu), wlying@mail.jlu.edu.cn (L. Wang).

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[3,4]. Synthetic non-methylated CpG-containing oligodeoxynucleotide (CpG ODN) possesses similar activity of stimulating innate immunity as bacterial dsDNA [3]. Based on the stimulatory signals, immune system makes a decision for setting effective responses to pathogens and tolerance to self-components so that it can sustain immune homeostasis. In a homeostatic condition, TLR activation leads to a protective immune response against pathogens through multiple inflammatory pathways. If an incorrect decision is made by immune system, a devastating consequence may occur to the host—shock in the short term or autoimmunity in the long term. In pathologic conditions, TLR9 can recognize self-DNA to initiate auto-innate immune responses [5]. Several reports show that TLR9 plays an important role in autoimmune disease development via activation by self-DNA [6,7]. CpG ODN-induced TLR9 activation was also reported capable of inducing a septic death due to excessive cytokine-mediated lethal shock in D-glactosamine (D-GalN) sensitized mice, in which massively released pro-inflammatory cytokines such as TNF-α and IL-12 were detected in the sera of the mice [3,8,9]. In recent years, with the outbreak of infection by severe acute respiratory syndrome coronavirus (SARS-CoV) and highly pathogenic H5N1 avian influenza virus, a cytokine storm due to TLR over-activation by viral infection is considered relevant to the high mortality [10]. It is thus clear that control of innate immune overreaction could be a new approach to prevent the development of TLR activation-associated diseases.

Different efforts have been made to develop agents for suppressing innate immune overreaction, providing a quite promising potential for clinical application on treating and preventing TLR activation-associated diseases. In recent years, DNA molecules have been found capable of inhibiting TLR activation [11]. In 2000, there was a report about DNA fragments derived from calf thymus and human placenta displayed an inhibitory role on IL-12 production of macrophage from mouse spleen and bone marrow [12]. Thereafter, an oligodeoxynucleotide (ODN) with mammal telomere-mimicking TTAGGG repeats, designated as A151, was reported to inhibit immune stimulatory activation by bacterial DNA and rescue mice from endotoxic shock by blocking LPS-induced production of IFN-γ and IL-12 [13]. A151 was also proved to have a therapeutic role in mice with severe arthritis induced by bacterial DNA, delay the onset of mouse glomerulonephritis and prolong the survival of lupus-prone NZB-NZW mice [14,15]. Another ODN with a guanosine-rich sequence (G-ODN) was identified to block the production of TNF-α and IL-12p40 by inhibiting TLR9 activation in a mouse model of D-GalN/CpG ODN-induced lethal shock [8]. Moreover, an ODN with a sequence of 5′-TGCTCCTGGAGGGGTTGT-3′, designated as IRS954, was revealed effective in reducing the severity of clinical symptoms in lupus-prone mice by antagonizing activation of TLR7 and TLR9 [16]. Structurally, all inhibitory ODNs introduced above are with G-rich and G-containing repeat sequences. However, it is still uncertain as to the key structural features for designing an efficient inhibitory ODNs, especially those that can select to suppress the activation of different TLRs.

In our laboratory, a series of microsatellite DNA (MS DNA)-mimicking ODNs (MS ODN) was designed according to microsatellite DNA repeats of human genome as inhibitory regulators of innate immune system. Human microsatellite DNA occupies about 5% of human genome and is highly repetitive elements composed of 2—6 nucleotides. An investigation indicated that genomic DNA derived from apoptotic or necrotic cells could be uptaken by macrophage and then cut into DNA fragments by DNase II, implying that abundant self-DNA fragments, a big part of them is from microsatellite DNA, have a big chance to stimulate host immune system. However, in physiological condition, these self-DNA fragments are unable to initiate anti-self-DNA immune response and immune-inhibitory possibly. To confirm the deduction, MS ODNs, ODN designed on the sequences of human microsatellite DNA, were observed for their inhibitory effect on immune responses of human PBMC to CpG ODN stimulation in vitro. It was found that MS ODN with T, TC, TTC, TTTC, TTCC, or TTTTTC repeats and also CT and CCT repeats could significantly inhibit CpG ODN-induced TLR9 activation [17], implying that human microsatellite DNA-derived DNA fragments may negatively regulate innate immune responses and therefore sustain tolerance to self-DNA.

In present study, an MS ODN with CCT repeats, designated as SAT05f, was studied for its inhibitory effect on TLR7/9 activation. It was found that SAT05f could down-regulate TLR7/9-dependent IFN-α production induced by viral nucleic acids in vitro, protect mice from D-GalN/CpG ODN-induced lethal shock but not TLR3/4 activation-mediated death, and suppress IFN-α production from human plasmacytoid cells (pDCs) induced by CpG ODN. Interestingly, SAT05f could also activate pDCs, manifested by up-regulating their MHC class II and costimulatory molecules. The data suggest that SAT05f could be used as a potential candidate for developing a medicament for the treatment of TLR9/TLR7 activation-associated diseases by blocking the TLR7/9 signaling.

Materials and methods

ODNs and reagents

Nuclease-resistant phosphorothioate-modified ODNs were synthesized in Takara Co. (Dalian, China). The CpG ODNs used in this study are 2216 (5′-ggGGG ACGATCGTCggggggG-3′), 1826 (5′-TCCATGACGTTCCTGACGTT-3′) and 1585 (5′-ggGGTCAACGTTGAgggggG-3′). The published inhibitory ODNs used in this study are A151 (5′-TTAGGGTTAGGGTTAGGGTTAGGG-3′) and IRS954 (5′-TGCTCCTGGAGGGGTTGT-3′). The own-designed ODNs are SAT05f (5′-CCTCCTCCTCCTCCTCCTCCTCCT-3′) and MS19 (5′-AAAGAAAGAAAG AAAGAAAGAAAG-3′). Lowercase and capital letters represent phosphorothioate and phosphodiester linkage, respectively. All ODNs were diluted in PBS and tested for endotoxin by using the Limulus amebocyte lysate assay (Associates of Cape Cod, Inc.). D-Galactosamine hydrochloride (D-galactosamine HCL, D-GalN) was purchased from DeBioChem (Nanjing, China). Chloroquine diphosphate salt, LPS, actinomycin D (ACTD), and polyinosinic—polycytidylic acid (poly I:C) were from Sigma (St. Louis, MO, USA). Imiquimod was from Lvye Chemical Co., Ltd. (Yancheng, China). Human recombinant IFN-α2b was from Neptunus Interlong Bio-technology Co., Ltd. (Shengzhen, China). All reagents used were pyrogen-free reagents.

Cells and cell lines

Human peripheral blood mononuclear cells (hPBMCs) were isolated from buffy coats (The Blood Center of Jilin Province,

China) by Ficoll-Hypaque density gradient centrifugation and washed three times with Iscove's modified Dulbecco's medium (IMDM, Hyclone, Logan, UT, USA). The viability of the PBMCs was 95–99% determined by trypan blue exclusion. Vero E6 cells (African green monkey kidney cell line; American Type Culture Collection (ATCC)) were cultured at 37 °C in a 5% CO_2 humidified incubator and maintained in IMDM supplemented with 10% (vol./vol.) heat-inactivated fetal bovine serum (FBS) and antibiotics (100 IU penicillin/ml and 100 IU streptomycin/ml). RAW264.7 cell (murine macrophage-like cell line; ATCC) and L929 cell (male BALB/C mouse fibroblasts) were cultured at 37 °C in a 5% CO_2 humidified incubator and maintained in RPMI 1640 medium supplemented with 10% (vol./vol.) heat-inactivated FBS and antibiotics (100 IU penicillin/ml and 100 IU streptomycin/ml). Human plasmacytoid dendritic cells (pDCs) were purified by Plasmacytoid Dendritic Cell Isolation Kit (130-092-207; Miltenyi Biotec, Bergisch Gladbach, Germany) stained by FITC-BDCA2 monoclonal antibody (BD Biosciences, San Jose, CA, USA) and analyzed on a FACS Canto (BD Biosciences) for the purity.

Preparation of virus

Influenza A virus Puerto-Rico-Stamm 8 (Flu virus PR8) [multiplicity of infection (MOI) = 3] was propagated in MDCK cells (Madin–Darby canine kidney cells; ATCC) cultured in IMDM, supplemented with 2% (vol./vol.) fetal bovine serum (FBS; Hyclone) and inactivated by heating at 56 °C in a water bath for 30 min. HSV-1 (MOI = 200) was propagated in Vero E6 cells cultured in IMDM supplemented with 2% (vol./vol.) FBS and then inactivated by heating at 70°C in a water bath for 10 min. VSV was grown in Vero E6 cells. After titration, the virus was stored in aliquots at −70 °C until use.

VSV protection assay

The antiviral activities in the culture supernatants of hPBMCs or mouse splenocytes treated by different stimulators or/and different inhibitors were measured using VSV protection assay. The supernatants of hPBMCs (5×10^5/well) cultured with different stimulators such as inactivated RNA virus (Flu virus PR8), DNA virus HSV-1, imiquimod and CpG ODN or/and different inhibitors such as A151, IRS954, SAT05f, MS19, and chloroquine for 48 h were collected and stored in aliquots at −70 °C until use. Vero E6 cells (2×10^4/well) or L929 cells (2×10^4/well) were seeded into 96-well flat-bottomed plates and cultured for 24 h to confluence. The cells were then incubated with 100 μl of the supernatants for 18 h and then challenged with 10× $TCID_{50}$ (50% tissue culture infectious doses) of vesicular stomatitis virus (VSV) for another 48 h. After discarding the culture supernatants, the cells were stained with 0.5% crystal violet and then analyzed for the cytopathic effect of the virus using Multi-well Microtiter Reader at $A_{570\ nm}$. The cytopathic levels of the cells were expressed as OD values.

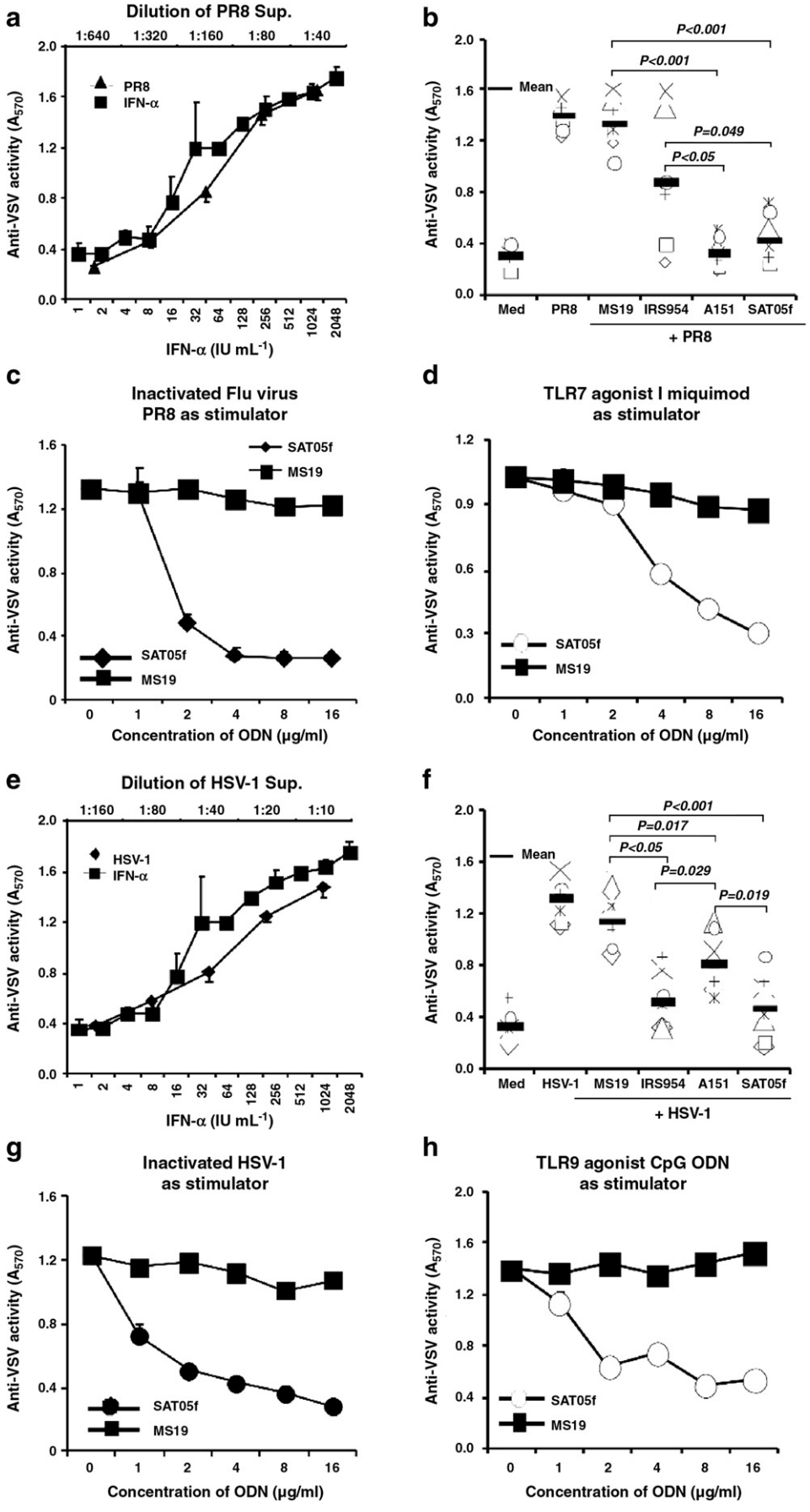
TNF-α bioassay

The activity of TNF-α in the supernatant was measured by an L929 cytotoxicity bioassay. This assay is based upon the cytotoxicity of TNF-α on L929 cells. Briefly, L929 cells were seeded at a density of 2×10^4 cells/well into a 96-well culture plate in RPMI 1640 medium containing 10% fetal bovine serum. After incubation for 24 h, the medium in the wells was replaced with the supernatant in doubling dilutions with the complete culture medium in the present of 1 μg/ml ACTD to incubate for another 24 h. After staining with 0.5% crystal violet, the cytotoxicity of TNF-α to the cells was determined by Multi-well Microtiter Plate Reader at $A_{570\ nm}$ and expressed as OD values. All experimental and control samples were performed in triplicate.

Mice and animal experiments

Eight-week-old specific pathogen-free (SPF) female BALB/C mice (20 ± 1 g weight) obtained from the Experimental Animal Center, Medical College of Norman Bethune, Jilin University were given free access to food and water during the experiments. For animal experiments, the mice were firstly injected intraperitoneally (i.p.) with 500 μl of D-GalN (32 mg/ml in PBS) for 1.5 h to make them become sensitive to immune stimulators and then randomly divided into different groups. CpG ODN 1826 (10 μg/per mouse in PBS), LPS (1 μg/per mouse in PBS) or poly I:C (50 μg/per mouse in PBS) were used as stimulators for triggering excessive cytokine-mediated lethal shock-like syndrome in mice by i.p. injection. ODNs, such as SAT05f and MS19, and chloroquine were also injected i.p. either simultaneously with stimulators or at different time point in mice. The survivals of the mice were recorded. All the experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals.

Figure 1 ODN-induced inhibition of RNA and DNA-mediated antiviral activity in vitro. Human PBMCs were stimulated with inactivated Flu virus (PR8) or DNA virus (HSV-1) for 48 h, and the supernatants were serially diluted for protecting Vero E6 cells from VSV challenge. The anti-VSV activity was paralleled with that displayed by recombinant IFN-α. (a and e) Inactivated virus-mediated anti-VSV activity: (a) Flu virus (PR8) and (e) DNA virus (HSV-1). (b and f) ODN-induced inhibition of inactivated virus-mediated anti-VSV activity. Human PBMCs were cultured in medium containing inactivated PR8 (b) or HSV-1 (f) with or without different ODNs (MS19, IRS954, A151, and SAT05f) for 48 h, and the supernatants were harvested for testing their VSV protection effect. Each symbol represents PBMC from one of seven donors. (c and d) Dose–effect curves of ODNs on inhibiting RNA-induced anti-VSV activity. Human PBMCs were cultured in medium containing inactivated PR8 (c) or Imiquimod (d) with or without different dosages of MS19 or SAT05f for 48 h, and the supernatants were harvested for assaying their VSV protection effect. Representative data from one of three donors are shown. (g and h) Dose–effect curves of ODNs on inhibiting DNA-induced anti-VSV activity. Human PBMCs were cultured in medium containing inactivated HSV-1 (g) or A-class CpG ODN (2216) (h) with or without different dosages of MS19 or SAT05f for 48 h and the supernatants were harvested for assaying their VSV protection effect. Representative data from one of three donors are shown. One symbol represents one donor-derived sample. $^{*}P<0.05$ and $^{**}P<0.001$ compared with virus-induced anti-VSV activities.


a
Dilution of PR8 Sup.
1:640 1:320 1:160 1:80 1:40
PR8
IFN-α
Anti-VSV activity (A570)
IFN-α (IU mL-1)
b
P<0.001
P<0.001
P=0.049
P<0.05
Mean
Med PR8 MS19 IRS954 A151 SAT05f
+ PR8
c
Inactivated Flu virus
PR8 as stimulator
SAT05f
MS19
Concentration of ODN (μg/ml)
d
TLR7 agonist I miquimod
as stimulator
e
Dilution of HSV-1 Sup.
1:160 1:80 1:40 1:20 1:10
HSV-1
IFN-α
f
P<0.001
P=0.017
P<0.05
P=0.029
P=0.019
Med HSV-1 MS19 IRS954 A151 SAT05f
+ HSV-1
g
Inactivated HSV-1
as stimulator
h
TLR9 agonist CpG ODN
as stimulator

Histological analysis of mouse livers

To analyze the pathological changes of mouse livers, liver organs of normal mice or D-GalN-treated mice injected with CpG 1826 or 1826 plus SAT05f were fixed in 10% neutral-buffered formalin and embedded in paraffin, cut into 5–10 μm thickness and then affixed to slides. The sections were stained with hematoxylin and eosin. The morphological changes on the sections of liver tissue were determined under microscopy.

Flow cytometry

Human pDCs (5×10^6 cells/ml) were incubated with or without CpG ODN 2216 (1 μg/ml) and/or SAT05f, A151, or MS19 at 8 μg/ml for 22 h, washed twice with FACS staining

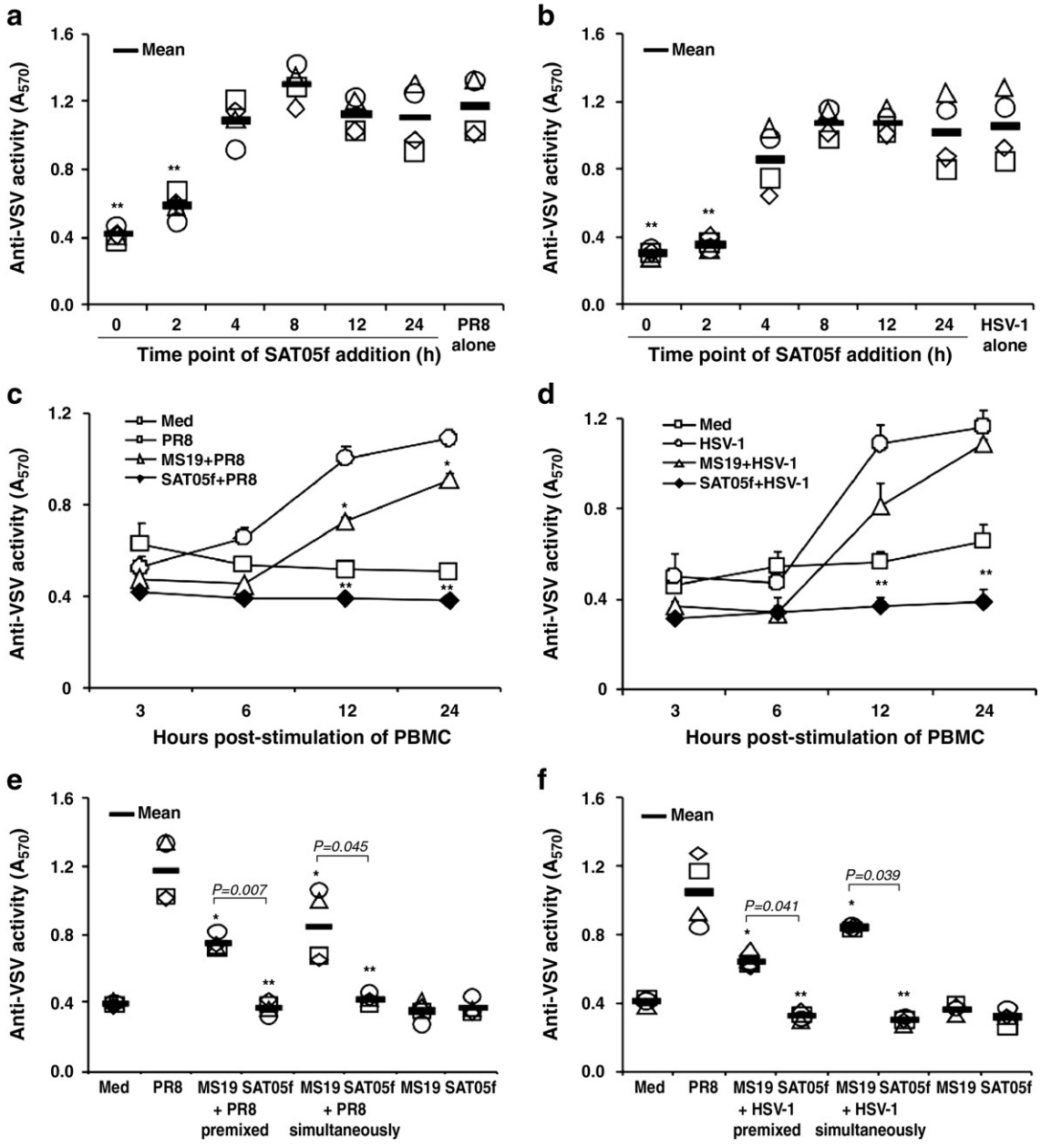


Figure 2 Kinetics of ODNs on inhibiting TLR7/9 agonists-mediated antiviral activities in vitro. (a and b) Time point of SAT05f addition to culture of human PBMCs. Human PBMCs were cultured in medium containing inactivated PR8 (a) or HSV-1 (b) with or without SAT05f added at different time points for 48 h, and the supernatants were harvested for VSV protection assay. (c and d) Time–effect curves. Human PBMCs were cultured with in medium containing inactivated PR8 (c) or HSV-1 (d) with or without SAT05f or MS19 for 3, 6, 12, and 24 h, and the supernatants were collected for VSV protection assay. (e and f) Compatible manner of ODNs and inactivated virus in culturing human PBMC. Inactivated PR8 (e) or HSV-1 (f) were either premixed with ODNs before addition or added simultaneously with ODNs to the culture of human PBMCs. The supernatants of the PBMCs were harvested after culturing 48 h and used for VSV protection assay. One symbol represents one donor-derived sample. $^{*}P<0.05$ and $^{**}P<0.001$ compared with virus-induced anti-VSV activities.

buffer (PBS supplemented with 0.5% BSA, 0.01% NaN_3, and 100 mM EDTA) after collecting the culture supernatants for cytokine detection and then analyzed for the expression of CD86, CD80, and HLA-DR by staining with PE-labeled anti-CD86, CD80, and HLA-DR mAbs (BD Biosciences, San Jose, USA) followed by analysis on a FACS Canto (BD Biosciences, San Jose, USA). The supernatants of the human pDCs were stored at −70 °C until use.

Binding and uptake assay of SAT05f

To analyze the binding and uptake of SAT05f, CAL-1 cells (2×10^6 cells/ml) were incubated with fluorescence Cy3-labeled SAT05f at 4 °C for 10 min or at 37 °C for 60 min. Then the cells were washed and analyzed by flow cytometry.

Cytokine detection by ELISA

The supernatants of the human pDCs stored at −70 °C were used for determination of cytokines. ELISA kits for IFN-α (Bender MedSystems, Inc., CA, USA), TNF-α and IL-6 (R&D System, Minneapolis, MN, USA) were used for detecting IFN-α, TNF-α, and IL-6 in the supernatants according to the manufacturer's protocols. The cytokine levels in the supernatants were quantitatively calculated based on the standard curve.

Statistical analysis

Data are shown as means±standard deviation (SD). The statistical significance of differences was determined using the paired two-tailed Student's *t*-test. Survivals of mice were compared by use of Kaplan–Meier test (the significance level was fixed at 0.05). Differences were considered statistically significant for $P<0.05$.

Results

An MS ODN with CCT repeats displayed an inhibitory effect on TLR7/9 activation in vitro

To develop a potential therapeutic agent for the treatment of autoimmune diseases, considering the close relationship between IFN-α/β production and the development of autoimmune diseases, we selected a type I interferon (IFN-α/β) bioassay, VSV protection assay, as a technical platform to screen the ODNs designed with the reference of the sequences of human microsatellite DNA for their inhibitory effects on type I IFN production. In the process, an MS ODN with CCT repeats, designated as SAT05f, was found to inhibit IFN-α production from cultured human PBMC stimulated by TLR7/9 agonists.

To determine whether or not SAT05f could block TLR7 activation, serial-diluted inactivated RNA virus (Flu virus PR8) as a TLR7 agonist was used to induce type I IFN production by human PBMC. The anti-VSV activities of the supernatants from TLR7 agonist-stimulated PBMC were compared with those displayed by recombinant IFN-α2b. As shown in Figure 1a, the Flu virus PR8-induced supernatants of human PBMC could protect Vero E6 cells from VSV challenge. An 80-fold-diluted Flu virus PR8 was able to induce the similar antiviral activity as 256 IU/ml of IFN-α2b did and used for later experiments. We then tested the inhibitory effect of SAT05f on IFN production by Flu virus PR8-induced PBMC. Human PBMCs were cultured with inactivated Flu virus PR8 in presence of SAT05f, or two positive control ODNs of A151 and IRS954, or a negative control MS ODN (MS19) fro 48 h and the supernatants were assayed for the anti-VSV activities. The result showed that SAT05f displayed significant inhibition on IFN production and its potency was similar as that displayed by A151 and much stronger than that by IRS954 and MS19 (Fig. 1b). To find an optimal dosage of SAT05f, PBMCs were incubated with inactivated Flu virus PR8 in the presence of MS19 or SAT05f

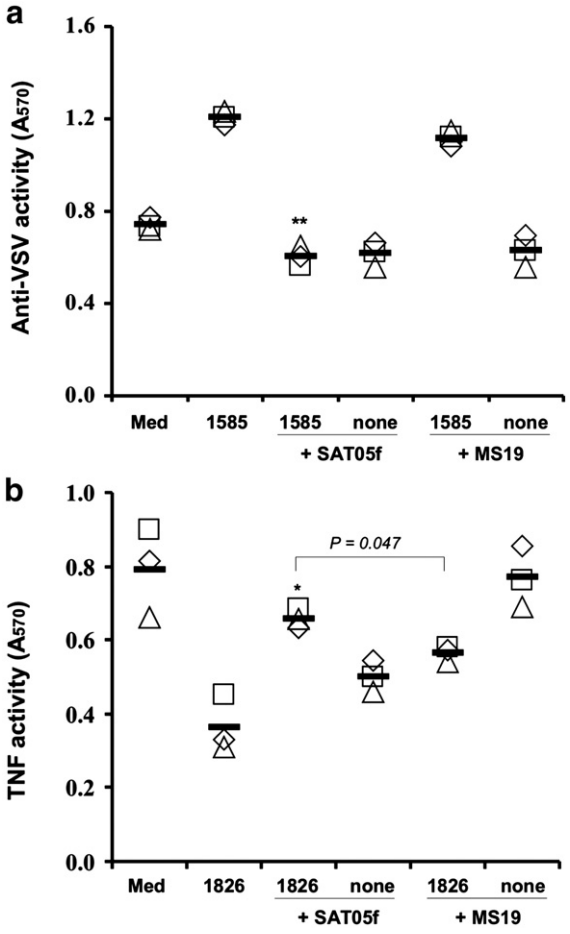
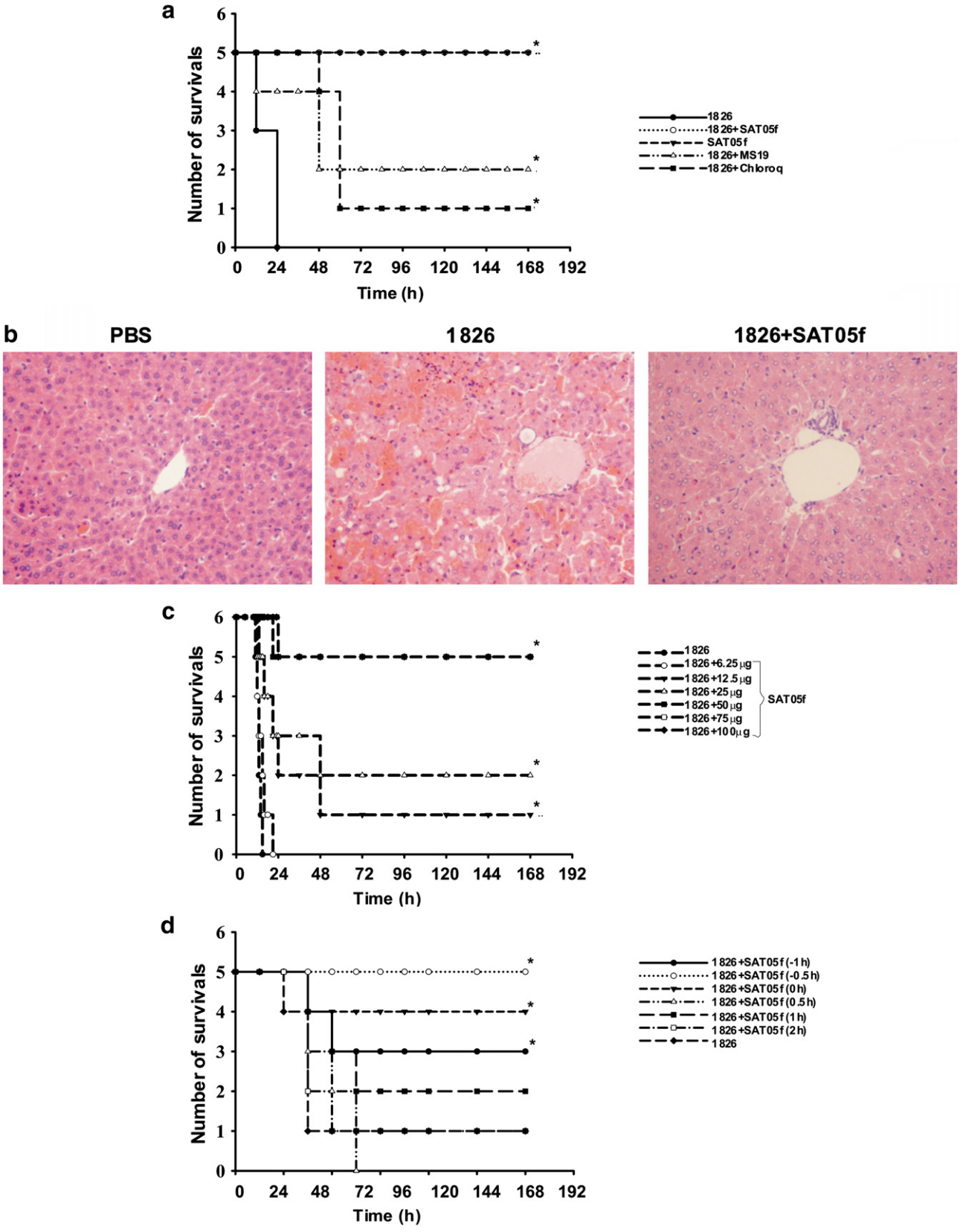


Figure 3 Inhibitory effect of SAT05f on TLR9 agonist-induced production of IFN and TNF in murine cells. (a) Effect of SAT05f on CpG 1585-induced IFN production from mouse splenocytes. Splenocytes from BALB/C mice were cultured in medium containing a mouse A-class CpG ODN (CpG 1585) or in the presence of SAT05f or MS19 for 48 h, and the supernatants were collected for protecting L929 cells from VSV challenge. One symbol represents the spleen cells derived from a mouse. (b) Effect of SAT05f on CpG 1826-induced TNF production from RAW264.7 cells. RAW264.7 cells (4×10^6/ml) were cultured in medium containing a mouse B-class CpG ODN (CpG 1826) or in the presence of SAT05f or MS19 for 8 h, and then the supernatants were harvested for assaying TNF-α activity by a TNF cytotoxicity bioassay method. One symbol represents one of three experiments. $^*P<0.05$ and $^{**}P<0.001$ vs. CpG 1585 (a) or CpG 1826 (b) alone.

at a dose range of 1–16 μg/ml for 48 h. It was found that PBMC from different donors responded to SAT05f variously and SAT05f at 8 μg/ml consistently inhibited IFN production from PBMC of all tested blood samples (data not shown). Upon this, SAT05f at 8 μg/ml was selected as the optimal dosage in later in vitro experiments. As shown in Figure 1c, SAT05f at 8 μg/ml remarkably inhibited Flu virus PR8-induced IFN production from PBMC of seven healthy blood donors ($p<0.001$),



indicating that SAT05f was an antagonist of TLR7 activation. In another set of experiment, inactivated Flu virus PR8 was replaced by imiquimod (a synthetic TLR7 agonist) as a stimulator, and SAT05f at dosages of 4–16 μg/ml could also display significant inhibitory effect on PBMC to produce IFN (Fig. 1d). Overall, these results showed that SAT05f could inhibit TLR7 activation-induced IFN production.

To test whether SAT05f could inhibit IFN production induced by TLR9 activation, inactivated DNA virus HSV-1 and CpG 2216 (an A-type CpG ODN) were used as stimulators. As shown in Figure 1e, 20-fold-diluted HSV-1 was able to display similar antiviral activity as 256 IU/ml of IFN-α2b did and used for later experiments. SAT05f was found to significantly inhibit inactivated HSV-1-induced IFN production from human PBMC with a much better efficacy than A151 (p=0.019) (Fig. 1f) in a dose-dependent manner (Fig. 1g). SAT05f could also inhibit CpG 2216-induced IFN production from human PBMC in a dose-dependent manner (Fig. 1h). These results indicate that SAT05f can also block TLR9 activation-induced IFN production.

Kinetics of SAT05f on inhibiting IFN production from PBMC induced by TLR7/9 agonists

To study the kinetics of SAT05f's action, human PBMCs were stimulated by either inactivated Flu virus PR8 or HSV-1, respectively, with or without addition of SAT05f at different time points. The culture supernatants were used for VSV protection assay. The result showed that SAT05f could significantly inhibit the IFN production from PBMCs activated by inactivated Flu virus PR8 and HSV-1 when added to the culture simultaneously with the stimulators or 2 h post-stimulation and failed to display obvious inhibitory role when added at 4 h or longer post-stimulation (Figs. 2a and b). This result illustrates that SAT05f may target same signaling molecules as TLR7/9 agonists and its affinity is not high enough to replace the stimulators once then acted on the signaling molecules.

Afterward, the supernatants of human PBMC cultured in medium containing TLR7/9 agonists with or without addition of SAT05f or MS19 were collected at different time points and assayed in VSV protection assay. It was showed that the supernatants from the PBMCs stimulated by inactivated Flu virus PR8 or HSV-1 for 12 h or longer could protect Vero E6 cells from VSV challenge but failed to display the protection if the supernatant was from the cells stimulated for less than 12 h (Figs. 2c and d). Upon this, the inhibitory effect of SAT05f on IFN production of human PBMCs triggered by TLR7/9 agonists was evaluated in 48 h after the stimulation.

To observe the influence of the ways of administrating SAT05f on its inhibitory effect, inactivated Flu virus PR8 or HSV-1 was premixed with SAT05f or MS19 for 2 h at 37 °C or used separately to act on human PBMCs for 48 h. The supernatants were assayed in VSV protection assays. The result showed that SAT05f and MS19, premixed or added simultaneously with inactivated Flu virus PR8 (Fig. 2e) or HSV-1 (Fig. 2f), displayed the similar inhibition on IFN production from human PBMCs. Comparatively, SAT05f displayed much stronger inhibition than MS19 (p<0.05) (Figs. 2e and f). The data indicate that SAT05f mediated inhibition is not due to its extracellular interaction with TLR agonists like Flu virus PR8 and HSV-1.

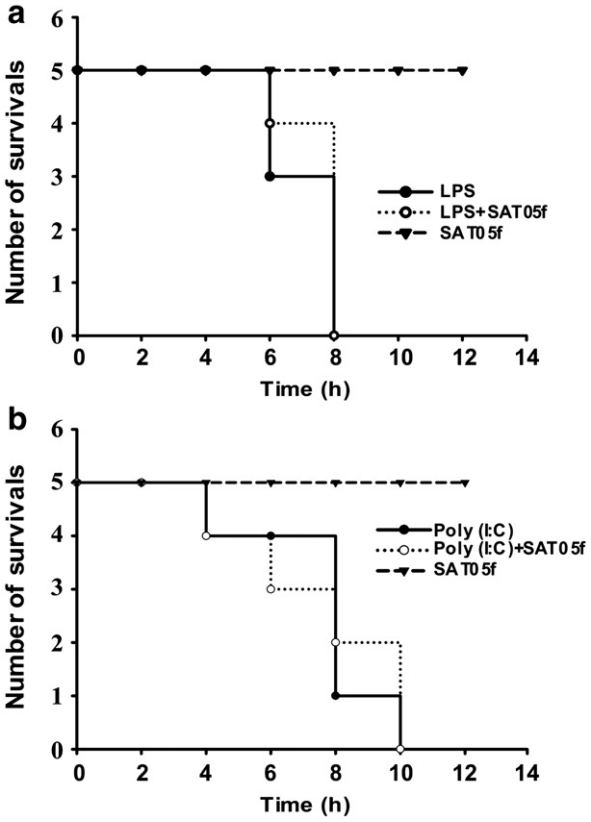
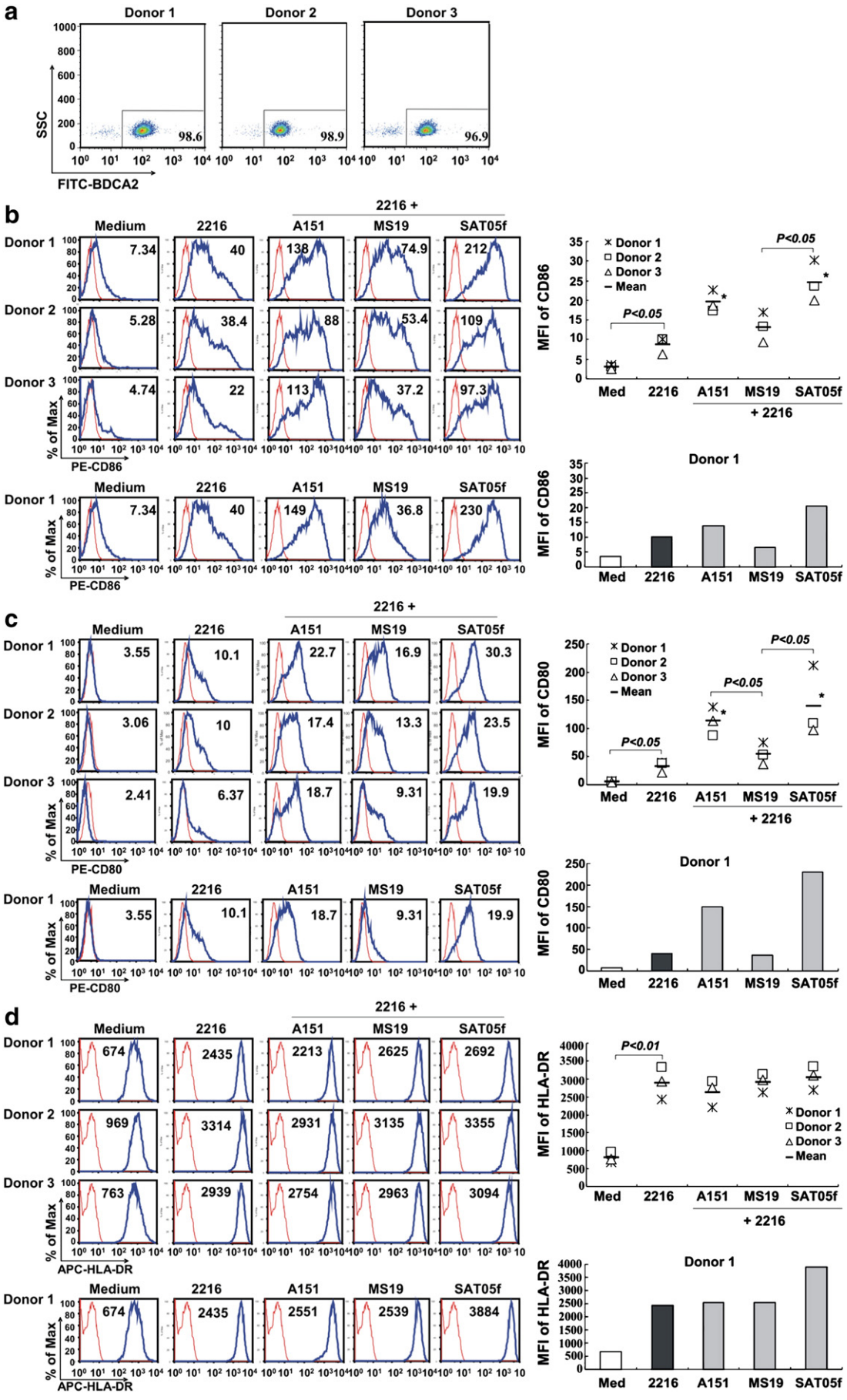


Figure 5 Effect of SAT05f on cytokine-mediated lethal shock induced by TLR4/TLR3 agonists in mice. BALB/C mice were injected i.p. with 500 μl of D-GalN (32 mg/ml) for 1.5 h and then treated by injection i.p. of LPS (1 μg/mouse)/poly (I:C) (50 μg/mouse) or SAT05f (250 μg/mouse) alone or both of LPS/poly (I:C) and SAT05f. Survivals of the mice were recorded. Representative data from one of three experiments are shown. (a) Effect of SAT05f on LPS-induced cytokine-mediated lethal shock in mice. (b) Effect of SAT05f on poly (I:C)-induced cytokine-mediated lethal shock in mice.

Figure 4 Effect of SAT05f on cytokine-mediated lethal shock induced by TLR9 agonist in mice. BALB/C mice were injected intraperitoneally (i.p.) with 500 μl of D-GalN (32 mg/ml) for 1.5 h followed by injection i.p. of CpG ODN (1826) at 10 μg per mouse, or SAT05f at 50 μg per mouse, or 1826 plus SAT05f, MS19 or chloroquine (1 mg per mouse). Survivals of the mice were recorded and histological sections of livers from the mice were prepared by H–E staining. $^{*}P$<0.05 vs. 1826-treated group. Representative data from one of three experiments are shown. (a) Survival curves of the mice. (b) Histological sections of mouse livers. (c) Dose–effect of SAT05f on cytokine-mediated lethal shock induced by 1826 in mice. (d) Kinetics of SAT05f on cytokine-mediated lethal shock induced by 1826 in mice.


a
Donor 1
Donor 2
Donor 3
SSC
FITC-BDCA2
98.6
98.9
96.9
b
Medium
2216
2216 +
A151
MS19
SAT05f
Donor 1
Donor 2
Donor 3
7.34
40
138
74.9
212
5.28
38.4
88
53.4
109
4.74
22
113
37.2
97.3
% of Max
PE-CD86
7.34
40
149
36.8
230
MFI of CD86
Donor 1
Donor 2
Donor 3
Mean
P<0.05
Med
2216
A151
MS19
SAT05f
+ 2216
c
3.55
10.1
22.7
16.9
30.3
3.06
10
17.4
13.3
23.5
2.41
6.37
18.7
9.31
19.9
PE-CD80
MFI of CD80
d
674
2435
2213
2625
2692
969
3314
2931
3135
3355
763
2939
2754
2963
3094
APC-HLA-DR
2551
2539
3884
MFI of HLA-DR
P<0.01

SAT05f rescued mice from excessive cytokine-mediated lethal shock

To study the inhibitory role of SAT05f on innate immunity in mice, we tested whether SAT05f worked functionally in mouse splenocytes. Firstly, BALB/C mouse-derived splenocytes were stimulated with CpG 1585, a typical mouse A-class CpG ODN, or in the presence of SAT05f or MS19 for 48 h, and the supernatants were used for protecting L929 cells from VSV challenge. As shown in Figure 3a, SAT05f could inhibit CpG 1585 to stimulate IFN production from the splenocytes whereas MS19 could not. We then detected whether SAT05f could block TNF-α production from mouse macrophages. A macrophage cell line RAW264.7 cells were stimulated with CpG 1826, a typical mouse B-class CpG ODN, or in the presence of SAT05f or MS19 for 8 h and the supernatants were harvested for assaying TNF-α production by a TNF-α cytotoxicity bioassay [18]. The result showed that SAT05f could inhibit the cytotoxicity of the supernatants from RAW264.7 cells triggered by CpG 1826 (Fig. 3b), demonstrating that SAT05f decreased the TNF-α production from mouse immune cells by suppressing TLR9 activation.

Upon the in vitro analysis, we then evaluated the in vivo effect of SAT05f in a mouse model of excessive cytokine-mediated lethal shock induced by different TLR agonists. To create the model, BALB/C mice were presensitized with D-GalN for 1.5 h and then injected i.p. with imiquimod and CpG 1826, respectively. In our system, imiquimod could not induce mice to develop the lethal shock even at 250 μg per D-GalN-sensitized mouse. Accordingly, D-GalN-sensitized mice were firstly injected with CpG 1826 or in the presence of SAT05f, MS19, or chloroquine (a TLR7/9 antagonist by interfering acidification of endosomes). The survivals of the mice were recorded. As apparently shown in Figure 4a, all mice in CpG 1826 group were dead in 24 h. In contrast, 100% of mice in CpG 1826 plus SAT05f group, 40% of mice in CpG 1826 plus MS19 group, and 20% of mice in CpG 1826 plus chloroquine group were still alive in 168 h, demonstrating that SAT05f could rescue mice from excessive cytokine-mediated lethal shock induced by TLR9 activation. Notably, MS19 could also partially resist CpG 1826-induced excessive cytokine-mediated lethal shock in mice whereas chloroquine was not remarkable as expected. Pathological analysis revealed that CpG 1826 was able to induce severe liver hemorrhage and necrosis in D-GalN-sensitized mice whereas SAT05f could prevent the pathological changes (Fig. 4b), implying that SAT05f displayed an inhibitory role of innate immune response to TLR9 activation and mediated a preventive effect for CpG ODN-induced liver injury in mice. Subsequently, the dose effect of SAT05f on resisting CpG 1826-induced lethal shock was observed in D-GalN-treated mice. As apparently shown in Figure 4c, all mice in groups of CpG 1826 alone or plus 6.25 μg of SAT05f were dead in 24 h, whereas 30%, 50%, and 80% of mice in groups of CpG 1826 plus SAT05f at 12.5 μg, 25 μg, and other three doses of 50, 75, and 100 μg were still alive in 168 h. Noticeably, SAT05f at 12.5 μg failed to protect mice from lethal shock induced by 10 μg CpG 1826. The molar ratio of 12.5 μg SAT05f and 10 μg CpG 1826 is 1.2:1, approximately. SAT05f at 50 μg consistently saved all tested mice (Fig. 4c). The molar ratio of 50 μg SAT05f and 10 μg CpG 1826 is 4.8:1, approximately. The data suggest that SAT05f could function by competing with CpG 1826. This result reveals that 50 μg of SAT05f may be an optimal dosage for rescuing mice from TLR9 activation-induced excessive cytokine-mediated lethal shock.

Moreover, we tested the suitable time for administration of SAT05f in D-GalN/CpG 1826-treated mice. SAT05f was separately injected i.p. into D-GalN sensitized mice at −1 h and −0.5 h before CpG 1826 injection, simultaneously with CpG 1826 injection or 0.5 h, 1 h, and 2 h after CpG 1826 injection. The survivals of the mice were recorded. The result showed that SAT05f could effectively protect mice from CpG 1826-induced excessive cytokine-mediated lethal shock when injected at −1 h, −0.5 h or simultaneously with CpG 1826 injection but failed to display the protection role when injected in 0.5 h or later after CpG 1826 injection (Fig. 4d). This may hint that SAT05f competed with CpG 1826 at the same site on target cells.

To know whether SAT05f could also inhibit activation of other TLRs, LPS (TLR4-ligand) and poly I:C (TLR3-ligand) were used as stimulators to induce excessive cytokine-mediated lethal shock in D-GalN-treated mice. The result showed that both poly I:C (50 μg/mouse) and LPS (1 μg/mouse) could induce death of D-GalN-treated mice. However, SAT05f could not rescue D-GalN-treated mice from LPS- and poly I:C-induced excessive cytokine-mediated lethal shock even at 250 μg (Figs. 5a and b). This may hint that SAT05f displays the inhibitory role of innate immune response by selecting TLR9 pathway but not TLR3 and TLR4 pathways.

SAT05f targeted immune cells for inhibiting TLR activation

Since pDCs are the major cells to produce IFN-α in response to TLR9 activation [19], we next tested whether SAT05f targeted pDCs to display its immune suppression. Human pDCs were purified from buffy coats of three healthy donors with >95% purity determined by FACS analysis (Fig. 6a). Afterward, CpG 2216 was selected as stimulator for pDCs because of its activation on immune cells to produce a large amount of IFN-α. The pDCs were stimulated with CpG 2216 alone or in the presence of SAT05f, A151, or MS19 for 22 h and then analyzed on a FACS Canto for their expression of surface molecules after staining with fluorescence-labeled mAbs. The result showed that the expression of CD86, CD80, and HLA-DR on the surface of pDCs was up-regulated not only

Figure 6 Effect of SAT05f on up-regulating surface molecules in human plasmacytoid dendritic cells in vitro. Human pDCs were purified from buffy coats of three different donors and analyzed for their purity with FITC-labeled anti-BDCA2mAb. The pDCs were cultured in medium containing CpG ODN (2216) with or without ODNs, or ODN alone for 22 h and then stained with PE-labeled anti-CD86, CD80, or HLA-DR mAbs, respectively, for FACS analysis (CD86/CD80/HLA-DR). (a) Analysis of pDC purity. (b) CD86 expression level on pDCs. (c) CD80 expression level on pDCs. (d) HLA-DR expression level on pDCs. Left are data from FACS analysis and right are data analyzed based on FACS analysis in panels (b), (c), and (d). $^{*}P<0.05$ and $^{**}P<0.001$ vs. medium.

by CpG 2216 but also by SAT05f or CpG 2216 plus SAT05f. The activities of SAT05f on up-regulating the surface molecules of pDCs were similar as that of A151 but much higher than that of MS19 (Figs. 6b–d). It was noteworthy that the lower activities of CpG 2216 on up-regulating CD80, CD86, and HLA-DR of pDCs could be due to its 1/8 dose compared other ODNs.

In another set of experiments, the effect of SAT05f on cytokine production from pDCs induced by CpG ODN was also observed. The purified pDCs were incubated with CpG 2216 or in the presence of SAT05f, A151, or MS19 for 22 h, and the supernatants were collected and assayed by ELISA. As shown in Figure 7, CpG 2216 itself could stimulate pDCs to produce high level of IFN-α, TNF-α and IL-6 and SAT05f, A151, or MS19 could not when used alone. When used together, SAT05f significantly inhibited IFN-α production from the pDCs triggered by CpG 2216. Comparatively, the inhibition displayed by SAT05f was more profound than that displayed by A151 (Fig. 7a). Obviously, SAT05f, displayed a tendency to, could not induce significant inhibition on the production of TNF-α and IL-6 from the pDCs treated by CpG 2216 (Figs. 7b and c). The result indicates that SAT05f targets human pDCs, cells expressing TLR7/TLR9, to control the production of IFN-α, possibly other cytokines induced via TLR9 activation.

SAT05f could bind to the surface of and be uptaken by its target cells

To observe if SAT05f could bind to the surface of its target cells, we labeled SAT05f at 3′-end with Cy3 and conducted a binding and up-taking assay in CAL-1 cells, a human plasmacytoid dendritic cell (pDC) line, using SAT05f-Cy3 (Cy3-labeled SAT05f). As described [20], the genetic and phenotypic features of CAL-1 cells bear a similarity to those of pDCs. CAL-1 cells prominently expressed not only TLR-7 and TLR-9, but also TLR-2 and TLR-4. After stimulation with TLR-9 ligands of CpG ODNs (ODN 2216), the cells produced TNF-α and IFN-α. This cell line should open the opportunity for study pDCs in vitro. With the reference of the paper [21], CAL-1 cells were cultured with SAT05f-Cy3 either at 4 °C for 10 min (for binding experiment) or at 37 °C for 60 min (for uptake experiment) at two doses of 1 μg/ml and 3 μg/ml, respectively, and then analyzed by flow cytometry. As shown in Figure 8, the fluorescence intensity of the cells cocultured with SAT05f-Cy3 either at 4 °C or at 37 °C was significantly increased in a dose-dependent manner compared to the medium control, indicating that SAT05f binds to the surface of and be uptaken by CAL-1 cells.

Discussion

In this article, we showed that a human microsatellite DNA-mimicking ODN (MS ODN) with CCT repeats (SAT05f) displayed an inhibitory role in TLR7/9 activation-induced innate immune responses possibly targeting pDCs.

Structurally, SAT05f was primarily different from the inhibitory ODNs reported previously. Both 2088 (5′-TCCTGG-CGGGGAAGT-3′) and 2114 (5′-TCCTGGAGGGGAAGT-3′) capable of blocking CpG-induced B cell activation have 2–3 G substitutions as their central motifs [22]. The suppressive activity of A151 (5′-TTAGGGTTAGGGTTAGGGTTAGGG-3′) was correlated with the TTAGGG repeats for forming G-tetrads [23]. IRS 954 (5′-TGCTCCTGGAGGGGTTGT-3′) was a G-rich ODN confirmed to inhibit IFN production by pDCs in response to DNA or RNA viruses [24]. Moreover, replacing GCGTT or ACGTT with GCGGG or ACGGG could convert a stimulatory 15-mer ODN into an inhibitory ODN [25]. Overall, all of the reported inhibitory ODNs are G containing or poly G containing or G-rich. Our previous study demonstrated that MS ODN with repeats composed of T or C could effectively display inhibitory effects on CpG ODN-induced innate immune responses in vitro [17]. SAT05f presented in this article has no "G base" and is composed of CCT repeats that are abundant in microsatellite DNA in human genome. Noticeably, to our knowledge, this was the first report showing that an oligonucleotide without "G base" is capable of inhibiting the activation of TLR7 and/or TLR9 and protecting mice from excessive cytokine-mediated lethal shock-like syndrome. This reveals that G-rich sequences are not the only structural feature for inhibitory ODNs. Recently, it has been reported that 2′ deoxyribose homopolymers with PS-modified backbone act as TLR9 and TLR7 antagonists. Addition of random DNA bases to the homopolymers does not alter their antagonist functions. The TLR9-inhibitory quality of the PS-modified ODN is overcome in the presence of a CpG motif because PS CpG ODN successfully converts the high TLR9 affinity of a PS-modified molecule into robust TLR9 activation [26]. These data supported that SAT05f should be inhibitory because it was a PS-modified ODN without CpG. However, our data challenge the view that random DNA bases do not affect the antagonist activity of PS 2′ deoxyribose homopolymers. For instance, PS-modified SAT05f could inhibit IFN production from in vitro cultured PBMC and pDCs induced by TLR agonists, whereas PS-modified MS19 was found almost inert on doing those. Moreover, SAT05f, not the MS19, inhibited TNF-α production from RAW264.7 induced by CpG 1826 and IFN production from BALB/c splenocytes triggered by CpG 1585. Interestingly, MS19 rescued mice from TLR9-dependent cytokine-mediated lethal shock, although less potent than SAT05f. These results indicate that the PS-modified ODN mediated inhibition does require particular motifs and the in vitro-inert PS-modified ODN might be inhibitory in vivo.

For investigating the inhibitory activity of SAT05f on innate immunity, TLR7/9 ligands were firstly selected as immune stimulators in our experiment system. Primarily, Flu virus PR8 (RNA virus) as a TLR7 agonist [2] and HSV-1 (DNA virus) as a TLR9 agonist [4] were used as major stimulators in in vitro experiments considering that TLR activation-associated "cytokine storm" was related to the high mortality during viral infection such as SARS and H5N1 avian flu [10]. We found that SAT05f could significantly inhibit human PBMC to produce type I IFN via TLR7/9 activation induced by inactivated Flu virus PR8 as well as HSV-1 and human pDCs to produce IFN-α triggered by a TLR9 agonist, indicating that SAT05f could show a promising prospect for developing a medication for controlling TLR7/9 activation-mediated innate immune overreaction such as the cytokine storm. The speculation was supported by our in vivo data that SAT05f could rescue the D-GalN-treated mice from excessive cytokine-mediated lethal shock triggered by TLR9 agonist.

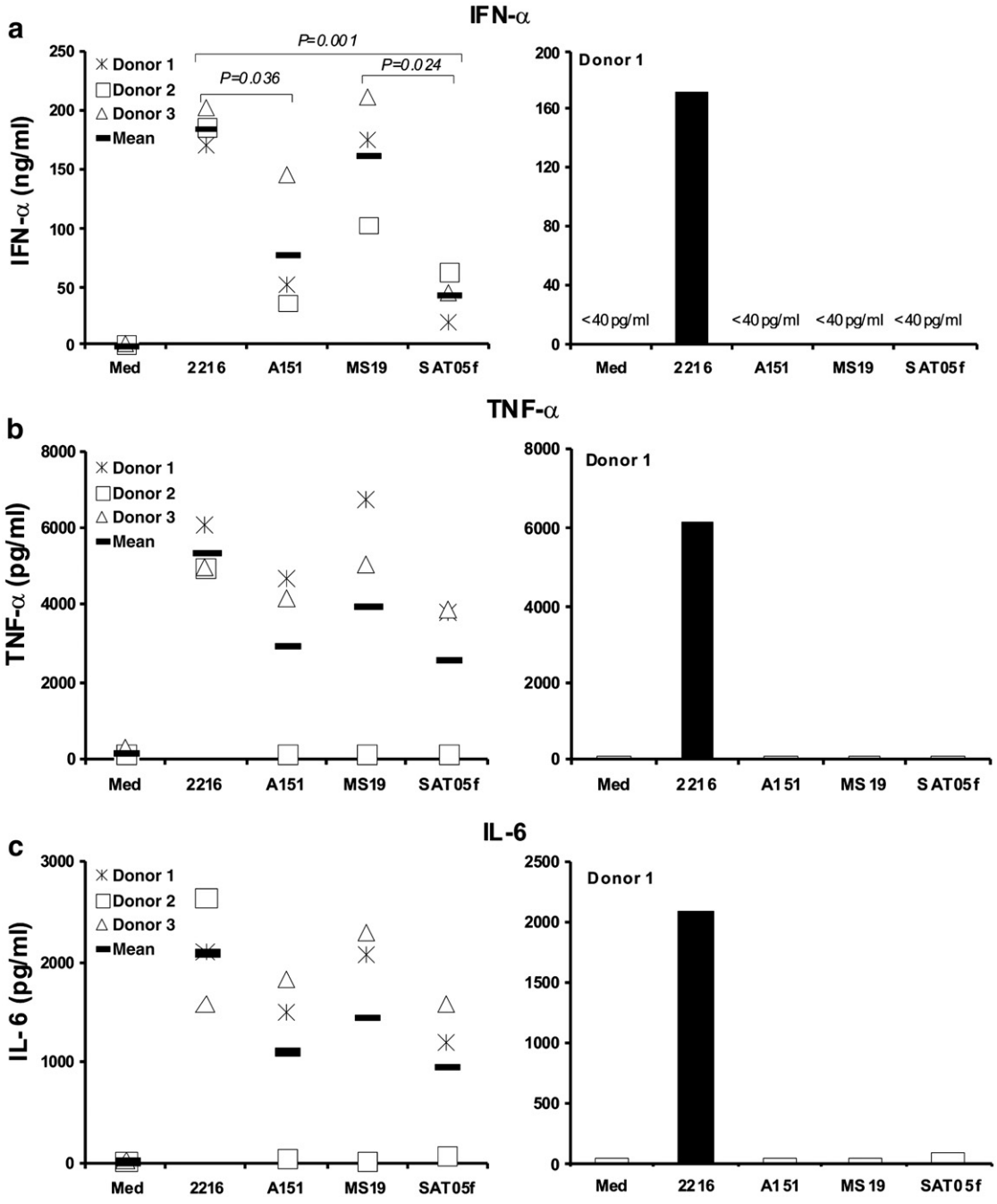


Figure 7 Effect of SAT05f on cytokine production of pDCs in vitro. Human pDCs were cultured in medium containing 1 μg/ml CpG ODN (2216) with or without ODNs (A151, SAT05f, or MS19) at 8 μg/ml or ODN alone for 22 h, and the supernatants were harvested for detecting cytokine production by ELISA. Each symbol represents cytokine level in pDCs supernatant from one of three donors. The left in the figure represents cytokine levels in the supernatants of cultured pDCs from all three donors, and the right in the figure represents cytokine levels in the supernatants of cultured pDCs from one donor. (a) IFN-α in the supernatants of cultured pDCs. (b) TNF-α in the supernatants of cultured pDCs. (c) IL-6 in the supernatants of cultured pDCs.

The involvement of TLR9 in the cytokine-mediated lethal shock was firstly revealed by the evidence that rapid TNF-α production from macrophages and toxic shock was induced in D-GalN sensitized mice by DNA prepared from Gram-positive/negative bacteria or synthetic oligonucleotides [27]. Our in vitro experiments showed that SAT05f inhibited TNF-α production from RAW264.7 cells, a BALB/C mouse macrophages cell line as well as from the pDCs from one blood donor. TNF-α was reported to induce excessive hepatocyte apoptosis once the cells were sensitized by D-GalN [28]. Pathological analysis showed that SAT05f protected mice from liver injury in D-GalN-sensitized mice induced by CpG ODN, implying that SAT05f might inhibit the development of excessive cytokine-mediated lethal shock-like syndrome by suppressing TNF-α-induced liver injury triggered by TLR9 activation [27,28]. In addition, TLR7 might also be involved

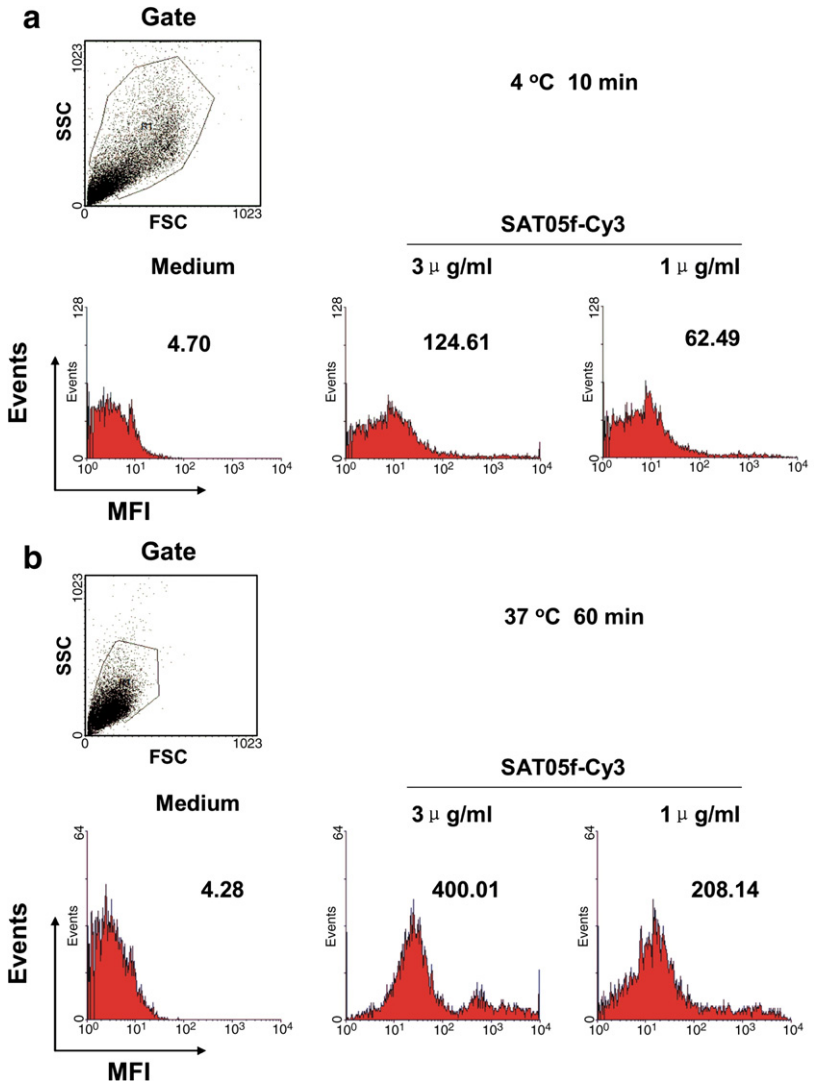


Figure 8 The binding and uptake of SAT05f in CAL-1 cell. CAL-1 cells were cultured in medium with or without SAT05f-Cy3 either at 4 °C for 10 min or at 37 °C for 60 min followed by washing three times and then analyzed by a FACScan. The result was expressed as mean fluorescence intensity (MFI). Representative data from one of three experiments are shown.

in the development of excessive cytokine-mediated lethal shock-like syndrome. Stimulation of TLR7 by H5N1 vaccine was found as the prime determinant of the greater magnitude of the vaccine-induced immune response [29]. In human cases infected with influenza A (H5N1) virus, excessive cytokine-mediated lethal shock-like syndrome characterized by higher plasma levels of cytokines has been observed in the patients, particularly in patients with fatal infection [30]. Considering that the possible involvement of TLR7 in influenza A (H5N1) virus induced hypercytokinemia and the data that SAT05f inhibited cytokine production induced by Flu virus PR8, a TLR7 agonist, we might speculate that SAT05f could inhibit the development of excessive cytokine-mediated lethal shock in influenza A (H5N1) virus-infected patients.

It has been established that CpG ODN and HSV-1 are TLR9 agonists [3,4] and that imiquimod and Flu virus PR8 are TLR7 agonists [2,31]. The data showed in this article demonstrated that SAT05f could significantly inhibit IFN production from PBMC stimulated with CpG ODN, HSV-1, imiquimod, and Flu virus PR8 and from purified human pDC stimulated with CpG ODN, indicating that SAT05f might be used as a TLR7/9 antagonist that down-regulates TLR9 or TLR7 activation-associated diseases accompanying high-level production of IFN. In recent years, TLR9/TLR7 pathways have been implicated in contributing to the development of inflammation-associated diseases and autoimmune diseases. A report showed that if TLR9-knocked out ($TLR9^{-/-}$) mice were used as graft recipients during allogeneic bone marrow transplantation, survivals and clinical scores of acute graft-vs-host disease (GVHD) were efficiently improved when compared with that in wild-type mice, suggesting that TLR9 activation contributed to the GVHD [32]. In a TLR9-dependent manner, immune complexes (ICs) consisting of autoantibodies specific to self-DNA and RNA induced pDCs to produce high level of IFN-α [7,33,34] that promoted the progress of systemic lupus erythematosus. Abnormal production of IFN-α from pDCs through TLR9 activation was involved in the development of psoriasis, Sjögren syndrome, type I diabetes, Hashimoto disease, and dermatomyositis

[35]. It was demonstrated that chronic triggering of TLR7 in mice using HIV-1-derived TLR7 ligands leads to immune activation and disruption of lymphoid architecture similar to those observed in chronic HIV-1 infection in humans [36]. Taken together, these findings also prompted us to develop SAT05f as a medicament for the treatment of TLR9/TLR7 activation-associated diseases by blocking the TLR7/9 signaling.

Our data in this article suggest that SAT05f functions by interfering with TLR signaling rather than by binding TLR ligands directly. Using a mouse model, we found that SAT05f, when administrated up to 1 h before stimulation with CpG 1826, still saved the mice from dying of excessive cytokine-mediated lethal shock-like syndrome. In addition to CpG ODN, SAT05f also inhibited IFN-α production induced by inactivated HSV-1, a TLR9 agonist and Flu virus PR8, a TLR7 agonist, obviously not by binding to the viruses directly. Analysis provided by other groups can also exclude the possibility. G-ODN, an inhibitory ODN, when administered up to 7 h after the stimulation with CpG. G-ODN still inhibited TLR9 activation-induced secretion of TNF-α and interleukin-12p40 and up-regulation of major histocompatibility complex (MHC) class II and costimulatory molecules [9]. Inhibitory ODN such as A151 and 2214, when added to endothelial leukocyte adhesion molecule gene transfected cells for 2 h prior to Cy3-labelled CpG ODN, could not interfered with the uptake of the CpG ODN [37].

Interestingly, we found that the SAT05f and A151, as CpG 2216, could up-regulate the expression of CD80, CD86, and HLA-DR on the surface of human purified pDCs. Seemingly, SAT05f-mediated inhibition on IFN-α production from pDC somehow depends on the up-regulation of these surface molecules on the pDCs. In other words, presumably, the inhibitory effect of SAT05f on IFN-α production results from the kind of activation of the pDCs.

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