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Human Serum Amyloid P Functions as a Negative Regulator of the Innate and Adaptive Immune Responses to DNA Vaccines

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The utility of DNA vaccines has been limited by their failure to elicit sufficiently potent immune responses in many human applications, whereas DNA vaccinations in mice have been very successful. However, the underlying mechanisms remain unknown. We hypothesize that serum amyloid P component (SAP), which has a species-specific, DNA-binding ability, contributes to the differences between human and mice and then limits DNA vaccine’s efficacy in vivo. In our study, DNA vaccine-induced adaptive immune responses were also significantly decreased in the human SAP (hSAP) transgenic mice. Using human promonocytic cell line THP-1–derived macrophages as a cell model, we found that cells incubated with a hSAP–DNA complex showed significant defects in innate immune activations, whereas mouse SAP had similar, albeit very weak, activities. hSAP also significantly inhibited the functions of two identified DNA sentinels, high-mobility group B protein 1 and antimicrobial peptide LL37, and redirected DNA update to FcRs leading to endocytosis and endosomal degradation. We also found that a chemical SAP inhibitor strongly recovered the suppressed innate immune responses to DNA in the presence of human serum and enhanced the immunogenicity of DNA vaccines in vivo. Our data indicated that SAP is a key negative regulator for innate immune responses to DNA and may be partly responsible for the insufficient immune responses after DNA vaccinations in humans. SAP suppression may be a novel strategy for improving efficacy of human DNA vaccines and requires further clinical investigations. The Journal of Immunology, 2011, 186: 2860–2870.

Over the last decade, the demonstration that plasmid DNA vaccines can induce both humoral and cellular immune responses to a variety of cancer and infectious diseases has engendered considerable excitement in the medical society (1–3). The speed of construction of DNA vaccines presents a real advantage for both prophylaxis and therapy of new pathogens, including emerging infectious agents, such as severe acute respiratory syndrome virus and emerging strains of influenza (2). Therapeutic vaccination is also available using DNA vaccines because they can offer a unique opportunity to guide defined Ags, accompanied by specific activator molecules, through a patient’s compromised immune system (4). In this regard, DNA vaccine holds enormous promise of vaccinologists and has entered into a variety of human clinical trials in the last decade.

However, despite its great success in mice-based investigations, the use of DNA vaccines has been greatly limited by their failure to elicit sufficiently potent immune responses in large animals and especially in human clinical trials (3, 4), and the underlying mechanisms that contribute to the differences in species-specific efficacy remain unknown. Accordingly, attempts have been made in recent years to find adjuvants that will enhance the immunogenicity of plasmid DNA vaccines (5). In our opinion, the identification of species-specific agents in the mechanism of DNA vaccines may be an urgent need to develop novel chemical adjuvant and is the main purpose of this study.

A fundamental initial and determined step in DNA vaccination is that the DNA vaccine needs to be transfected into various types of cells in situ and then result in subsequent Ag expression and innate immune activation in vivo (1). It is well-known that there are abundant physiological circular DNA-binding proteins in the extracellular environment. Certainly these proteins first meet the administered plasmids, and we therefore hypothesize that some of them may contribute to the insufficient efficacy of DNA vaccine. Factually, recent studies have indicated that several extracellular DNA-binding proteins, such as high-mobility group B protein (HMGB) (6), antimicrobial peptide LL37 (also known as CAMP) (7), and heparan sulfate proteoglycan (8), may play a key role in DNA uptake under physiological conditions (6, 7).

Another important mechanism of DNA vaccines is that DNA potently activates the innate immune responses through various DNA sensors such as endosomal TLR9 (9) and cytosolic DNA-sensing receptors (10–15), and the innate activation is crucial to the immunogenicity of DNA vaccines (11). Interestingly, it was also recently shown that the extracellular DNA-binding proteins can act as nucleic acid sentinels and convert extracellular DNA
into a potent trigger of innate immune activation (16-19), which subsequently breaks innate tolerance to DNA and may drive many autoimmune diseases (20). For example, HMGBs function as universal sentinel for all immunogenic nucleic acids (6, 16, 21). LL37 binds and delivers DNA into plasmacytoid dendritic cells to trigger TLR9 (7). All those recent advances about extracellular DNA-binding proteins provide novel directions to study the underlying mechanism of DNA vaccine, which gives us a new hope to develop optimal vaccines for clinical applications.

However, it remains unknown whether an extracellular DNA-binding protein exists that negatively regulates the DNA uptake and innate immune recognition. In this study, we hypothesize that certain extracellular molecules may facilitate the clearance of extracellular DNA and form barriers that prevent the immune responses to DNA vaccines. To identify the potential barrier molecules, we previously scanned and identified the DNA-binding proteins found in human and mouse serum. Among those identified proteins, human serum amyloid P component (hSAP) was the most intriguing protein. First, SAP is well-known as the major genomic binding protein in human plasma (22), but mouse SAP (mSAP) has a much weaker ability to bind DNA (23). Furthermore, SAP is a member of the short pentraxin family, which is constitutively present in human serum at 30 to 50 μg/ml and is a normal component of basement membranes (24, 25). At the same time, many reports have also shown that SAP can bind apoptotic cells and zymosan in a calcium-dependent manner and enhance their phagocytosis by macrophages (25, 26). In this regard, we believed that hSAP is a candidate DNA vaccine “scavenger.” However, the role of hSAP in innate immune recognition of DNA and the underlying molecular mechanisms remain undefined and require further investigations.

Because the interventions cannot be tested in humans for ethical reasons, in this study, we used a humanized mouse model, a transgenic (Tg) mouse strain physiologically expressing hSAP, to confirm our hypothesis. We also investigated the molecular and cellular mechanisms of hSAP-mediated immune suppression in vitro and in vivo. Based on our data, we demonstrated a fundamental and important role of a circulating pentraxin in the negative regulation of immune recognition of DNA vaccine. We suggest that SAP may be partly responsible for insufficient immune responses after DNA vaccination in humans and further suggest its potential implications for improving DNA vaccine efficacy.

Materials and Methods

Mouse and cells

hSAP Tg mice (Tg[Alb, SAP]; C57BL/6, H-2b) were generated by Model Animal Research Center of Nanjing University (China), which contains the entire hSAP open reading frame under transcriptional control of the mouse albumin promoter and produces high-level hSAP in serum. C57BL/6 mice were obtained from the Shanghai Laboratory Animal (China). The HEK293 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen). THP-1 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. PMA (10 μg/ml; Sigma-Aldrich, St. Louis, MO) was added into the THP-1 medium to induce macrophage differentiation, and the cells were cultured for 3 d with daily medium replacement.

Reagents

hSAP and mSAP were purified, stored, and dialyzed as previously described (27, 28). They were stored frozen at −70°C in solution in 10 mM Tris, 140 mM NaCl, 10 mM EDTA, 0.1% NaN3, pH 8.0 (Tris-sodium-EDTA buffer), and were dialyzed into 10 mM Tris, 140 mM NaCl, pH 8.0 (Tris-sodium buffer) before use. The peptide LL37 (LLGDFPFRKKSKERGKPKYDQKRKDLRNLYPST) was synthesized and biotinylated by Sangon Biotech (Shanghai, China). A SAP inhibitor CHPPCH (R-I-[6-[2-carboxy- pyrrolidin-1-yl]ethoxy-hexanoyl]pyrrolidine-2-carboxylic acid) was synthesized according to previous reports (29). Human C-reactive protein (CRP) was obtained from Sigma-Aldrich, and Texas Red-conjugated human transferrin was from Rockland (Gilbertsville, PA). A monospecific Ab for hSAP (Abcam, Cambridge, MA) and a goat polyclonal Ab both for hSAP and mSAP (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Other Abs for early endosome Ag 1, CD107a (BD Pharmingen, San Diego, CA), spleen tyrosine kinase (Syk), phospho-Syk (Tyr525/526), Rab5 (Cell Signaling Technology, Danvers, MA), IFN regulatory factor 3 (IRF3), and C1q Ab (Abcam) were all obtained from commody.

Plasmids and other DNA ligands

All oligonucleotides were synthesized by Sangon Biotech (Shanghai, China), with p-ho linkages represented as lowercase; IFN-stimulating DNA (ISD) (sense), 5′-TACGATCTACTAGTGATCTATGACTGATCT- GTACGATCTACCA-3′; CpG-5′-TCCATAGCTGTCCCTGAGCTT-3′. Equimolar amounts of ISD and their antisense oligos were annealed in PBS at 75°C for 30 min before cooling to room temperature. ISD-reverse was the exact reverse sequence of that listed earlier. poly(dA-dT)poly(dT-dA) was obtained from Sigma-Aldrich. Plasmid pGL4.17 was obtained from Promega (Madison, WI). DNA vaccine pVAX-HBs was constructed by inserting a cDNA fragment encoding hepatitis B virus surface Ag (HBsAg) into pVAX1 (Invitrogen) in our previous studies (30). All plasmids were isolated using an Endo-free Plasmid Mega Kit (Qiagen China, Shanghai, China). For confocal analysis, DNA was labeled with FITC using Label-it Fluorescence Kit (Mirus Bio, Madison, WI) as recommended by the manufacturer.

Cytokines measurements

Cytokines mRNA was determined by quantitative RT-PCR (primers pairs shown in Supplementary Table I) with Step-one Plus Real-Time PCR Systems (Applied Biosystems, Carlsbad, CA). TNF-α, IL-6, and IFN-β protein expression level was also determined by ELISA (BD Pharmingen).

Evaluation of transcription factor activity

For IRF3 activation, whole-cell lysates from monocyte-derived macrophages (MDMs) stimulated for the indicated times were separated by SDS-PAGE or native PAGE, transferred to polyvinylidene difluoride membranes, and probed with Abs specific for the indicated proteins. For NF-κB activation, EMSA was performed with standard techniques; the sequence of NF-κB probe (sense) is 5′-AGT TGA GGG GAC TTT CCC AGG C-3′. NF-κB activation was also quantified by way of ELISA using the TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA), according to the manufacturer’s instructions.

Protein-DNA binding assays

For immunoprecipitation, HMGB1 (1 μg) and LL37 (1 μg) were each incubated with SAP (1 μg) protein in Tris-sodium-calcium (TSC) buffer (10 mM Tris, 140 mM NaCl, 2 mM CaCl2, 1% NaN3, pH 8.0) for 30 min and then incubated with anti-SAP Ab for 30 min. The complexes were immunoprecipitated with G UltraLink Resin or Streptavidin UltraLink Resin as a control (Pierce Biotechnology, Rockford, IL). For the competitive EMSA, biotin-labeled LL37 (1 μg) and ISD (0.2 μg) were incubated with increasing amounts of SAP and then separated on an 8% native PAGE gel in 45 mM Tris-borate buffer (without EDTA, pH 8.0). The biotin-labeled LL37 migration was visualized after HRP-labeled streptavidin staining. For the competitive ELISA, HMGB1 (1 μg) or LL37 (1 μg) was coated onto 96-well plates, and binding of biotin-labeled ISD was detected with HRP-labeled streptavidin (KPL, Gaithersburg, MD). In the inhibition assay, SAP was incubated with biotin-labeled ISD with or without 10 nM CHPPCH, which was then added to coated plates.

DNA uptake and stimulation in vitro

Uptake of FITC-labeled plasmid by cells was determined by FACs analysis as described previously (31). To remove extracellular DNA, we washed cells three times with PBS containing dextran sulfate (100 μg/ml). In some cases, cells were treated with methyl-β-cyclodextrin (1 mM), amiloride (2.5 mM), wortmannin (20 μM), latrunculin A (2.5 μM), piceatannol (25 μM), polyguanylic acid (polyG; 500 μg/ml; all from Sigma-Aldrich), soluble IgG (10 mg/ml IVig; Shanghai Institute of Biological Products), and soluble advanced glycosylation end product-specific receptor (sRAGE; 5 μg/ml; BioVendor Laboratory Medicine, Czech Republic) for 30 min before incubation and during uptake experiments. The peptides LL37 (40 μg/ml), HMGB1 (10 μg/ml; GenWay Biotech, San Diego, CA), and hSAP (40 μg/ml) were individually preincubated with FITC-labeled DNA (8 μg/ml) for 10 min in TSC buffer before being added to the culture.
Confocal microscopy

MDMs were differentiated on glass coverslips in 24-well plates as described previously (26). hSAP (10 μg/ml) and FITC-labeled DNA (8 μg/ml) were preincubated in TSC buffer at 37˚C for 10 min and then incubated with cells at 37˚C, 5% CO₂ for 5 or 15 min. The cells were then fixed and stained using anti-SAP mAb and other Abs. In some cases, Texas Red-conjugated transferrin (10 μg/ml) was also added into the medium as recommended by the manufacturer. For chloroquine treatment, 100 μg/ml chloroquine was administered in cell culture for 30 min and then removed by PBS washing. An inverted Leica TCS SP2 AOBs confocal microscope (DMIREFER; Wetzlar, Germany) with an HCX PL APO lbd. BL 63×/1.4 oil immersion objective was used. Images were collected with Leica Imaging Software (CTRMIC; TCS SP2). Representative images were processed in Photoshop (Adobe Systems, San Jose, CA) applying only linear corrections.

SAP-DNA binding analyses

The binding of hSAP to DNA was evaluated using dot blot and gel retardation analysis as previously described (32). For dot blot, all DNA blots were incubated with 10% human serum diluted with TSC buffer for 30 min and then visualized with a SAP-specific Ab. For gel retardation analysis, DNA (0.2 μg) was incubated with purified SAP or 5% serum in TSC buffer for 10 min and then separated on 5% native PAGE in 45 mM Tris-borate buffer (without EDTA, pH 8.0). DNA migration was visualized after ethidium bromide staining on a UV board. Three pGL4.17-derived DNA fragments used in gel retardation analysis were obtained by PCR.

DNA cloning and transfection

Full-length cDNA of FcγRIa, FcγRIIa, FcγRIIb, FcγRIIIa, scavenger receptor A (SRA), and advanced glycosylation end product-specific receptor (AGER) genes were obtained using RT-PCR from THP-1 total RNA and then inserted into pcDNA3.1(−)-flag (Invitrogen). The expressing plasmids were transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and the expression levels of these receptors were confirmed by RT-PCR.

Animal experiments

Mice were maintained under specific pathogen-free conditions, and the experimental protocols were approved by the Second Military Medical University Institutional Animal Care and Use Committee. For immune stimulation in vivo, mice were intravenously injected with B-form DNA (B-DNA; 5 μg/g), and sera were collected by tail bleeding immediately or 6 h after injection. For DNA vaccine immunization, 50 μg pVAX-HBs was inoculated into the quadriceps every 2 wk three times. In some cases, CHPHC (50 μg/g) was also involved in the formulation of B-DNA or DNA vaccines.

Immunossays

HBsAg-specific Ab analysis, spleen Ag-specific IFN-γ production, and Ag-specific CTL activity was performed as we previously described (11, 30). In brief, sera from the immunized mice were collected by tail bleeding 2 wk after last immunization for anti-HBsAg Ab ELISA assay. For the Ag-specific CTL activity, splenocytes from each mouse were separated at week 1 after the final immunization. A murine melanoma cell line B16 (H-2b) stable expressing HBsAg (B16/S cell) was used as target cells for HBsAg-specific CTLs. After a stimulation of splenocytes with peptides for 7 d in vitro, 1×10⁵ CTL effector cells were cocultured with 2×10⁵ B16/S cells in 200 μl medium per well for 4 h. Cytotoxic activity of CTL was tested by a colorimetric assay (Roche Applied Science, Mannheim, Germany), which is based on the measurement of lactate dehydrogenase activity released from the cytoplasm of damaged cells, and the percentage of specific lysis was calculated as described previously (30). For the specific IFN-γ assays, bulk splenocytes were then cultured for 24 h at 37˚C with 5% CO₂ in the presence of 10 μg/ml peptides. Then the supernatant was collected and determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN).
Systems, Minneapolis, MN) according to their manuals. The stimulating peptide is the 8-mer peptide ILSPFLPL (HBsAg S208-215) (33).

**Statistical analysis**

Differences between the groups were analyzed for statistical significance by the two-tailed unpaired Student t test, using SPSS 11.5 software.

**Results**

**hSAP attenuates the immunogenicity of HBsAg DNA vaccine in vivo**

In our study, we first confirmed the DNA-binding ability of hSAP and mSAP with various kinds of DNA, and our data indicated a length- and sequence-independent universal DNA-binding ability of hSAP (Supplemental Fig. 1). Consistent with previous reports, purified mSAP did not show a significant DNA-binding ability (Supplemental Fig. 1), which also confirmed the species-specific DNA-binding ability of SAP. We also found that hSAP’s DNA-binding ability was strongly inhibited by the presence of CHPHC (a SAP-specific inhibitor that cross-links and dimerizes SAP molecules) (29) (Supplemental Fig. 1C), which could be a potential chemical inhibitor for hSAP in this study.

Basing on these results, we then investigated the effort of hSAP on the immunogenicity of DNA vaccines in vivo using a humanized mouse model expressing hSAP (Supplemental Fig. 2). We used an HBsAg-producing plasmid (pVAX1-HBs) as a model vaccine for i.m. inoculation into the hSAP Tg mice. When wild-type (WT) mice were immunized with pVAX-HBs, their IgG titers against the encoded HBsAg protein were significantly augmented in serum; however, titers in hSAP Tg mice were significantly lower than those in the WT mice (Fig. 1A). The isotypes of HBsAg-specific IgG had a similar change (Fig. 1B). For cellular immunity, the secretion of IFN-γ by splenocytes (Fig. 1C), and the frequency and cytotoxicity of Ag-specific CD8+ T cells (Fig. 1D) from hSAP Tg mice was also significantly lower than those from the control groups. These results demonstrate that hSAP Tg mice elicited significantly weaker humoral and cellular immune responses than WT mice. We also found that HBsAg-specific immune responses in CHPHC-administered hSAP Tg mice reverted to the level observed in WT mice. We therefore suggest that hSAP may inhibit the immunogenicity of HBsAg-expressing DNA vaccines in vivo, and the SAP inhibitor may play a role in the improvement of DNA vaccine efficacy.

**hSAP inhibits DNA-mediated innate immune responses in vitro**

We then wondered about the mechanism of hSAP-induced suppression to DNA vaccines. Previous studies have shown that plasmid-DNA–activated innate immune responses and the resultant type I IFN-mediated signaling is necessary for the optimal immunogenicity of DNA vaccine (11). Recent studies have

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**FIGURE 2.** hSAP inhibited DNA-mediated innate immune activation in vitro. A–C, THP-1 MDMs were stimulated with CpG-B and B-DNA for 6 h in the presence of hSAP, mSAP, or nothing. The relative expression level of cytokine mRNA was determined by quantitative real-time RT-PCR. The relative expression levels were assayed using the \( \Delta\Delta^Ct \) method and are presented as mean values and SD based on three independent experiments. *p < 0.05, **p < 0.01 compared with DNA-stimulated MDMs (two-tailed unpaired t test). D, DNA-hSAP complexes were incubated with MDMs, and IRF3 and NF-κB activation was determined with Western blotting and EMSA, respectively. ND, not detected.
also shown that aggregated SAP alone can stimulate the productions of IL-10, IL-8, and IL-6 by CD14+ monocytes via the FcγRIIa–Syk signaling pathway (26). We therefore wanted to know whether SAP–DNA complexes can also induce innate immune activation. In this regard, we examined macrophage cytokine production after DNA–SAP complex stimulation using human promonocytic cell line THP-1–derived macrophages (MDMs) as cell models, which express various DNA sensors and strongly respond to immune-stimulating DNA, including TLR9 agonist oligonucleotides with p-thio linkages (CpG-B) and B-DNA such as ISD and poly(deoxyguanylic-deoxythymidylic) acid. The hallmark of the innate immune responses activated by these receptors is the induction of type I IFNs, proinflammatory cytokines and chemokines (6). Interestingly, we found that MDMs in the presence of hSAP showed no augmented activation but showed a significant defect in mRNA induction for type I IFNs and a chemokine CCL5 in response to extracellular B-DNA at all doses examined, whereas the MDM response to DNA remained unaffected in the presence of mSAP (Fig. 2A). Similar results were also obtained on stimulation of MDMs with CpG-B in the presence of hSAP or mSAP (Fig. 2B).

At the same time, we also found that an immune inhibitory cytokine, IL-10, was significantly increased after hSAP-DNA stimulation (Fig. 2C) and was previously reported to be induced by aggregated SAP (26). All of these data suggest that hSAP inhibits DNA-mediated immune activation.

We next examined whether the signaling pathways activated through the cytosolic receptors are affected by hSAP. The activation of the transcription factors IRF3 and NF-κB was measured in MDMs stimulated by B-DNA or CpG-B in the presence of hSAP. As shown in Fig. 2D, the formation of dimerized IRF3, a hallmark of IRF3 activation, was strongly suppressed in hSAP-DNA–stimulated MDMs but robust in normal MDMs on stimulation with ISD. Similarly, the activation of NF-κB was also suppressed in hSAP-DNA–stimulated MDMs (Fig. 2D). Thus,
these results indicate that, in the context of the DNA-mediated activation of innate immune responses, hSAP plays an inhibitory role in the cytosolic receptor–IRF3/NF-κB signaling pathways.

**hSAP competitively inhibits the DNA-binding ability of HMGB1 and LL37**

Previous studies have shown that extracellular DNA-binding proteins and peptides, such as HMGB1 and LL37, can facilitate DNA uptake and contribute to DNA innate immune recognition. However, our results suggested that another extracellular protein, SAP, may play a role as an inhibitory protein. It was therefore important to determine how these extracellular DNA-binding proteins interacted. We investigated the DNA-sentinel attributes of HMGB1 and LL37 in the presence of SAP. As shown in Fig. 3A, IFN-β protein secretion after stimulation with B-DNA was significantly lower in the presence of hSAP than in the presence of mSAP or the control protein human CRP. This inhibition can also be abolished using the SAP inhibitor CHPHC, which further suggests the requirement for a natural pentamer structure during the process. We next examined whether the signaling pathways activated through the HMGB1 or LL37 and cytosolic DNA-Rs are affected by hSAP. Activation of the IRF3 and NF-κB transcription factors was measured in MDMs stimulated by ISD or CpG-B. hSAP strongly suppressed the formation of dimerized IRF3 in either HMGB1– or LL37–ISD complex-stimulated MDMs (Fig. 3B). Similarly, the DNA-binding ability of NF-κB was also suppressed in the presence of hSAP (Fig. 3C). These results indicate that, in the context of the nucleic-acid–mediated activation of innate immune responses, SAP negatively regulates the cytosolic receptor–IRF3/NF-κB signaling pathways.

**FIGURE 4.** hSAP facilitates plasmid DNA endocytosis. FITC-labeled B-DNA or CpG-B was coincubated with hSAP or other proteins and then was added to THP-1 MDMs for 5 min, 30 min, or 4 h. In some cases (A, C–E), MDMs were treated with chloroquine (100 μg/ml) before DNA incubation. The intracellular DNA was determined with flow cytometry (A, B, E), C and D, MDMs were stained with Abs and observed with confocal microscopy. All samples were tested in duplicate, and the colocalization results were shown as the representative pictures. E, hSAP facilitates DNA endocytosis, whereas LL37 facilitates DNA macropinocytosis. MDMs were respectively treated with the shown inhibitors before incubation. The FITC-labeled B-DNA was used and the fluorescence-positive cell rates are shown as mean and SD based on three independent experiments. *p < 0.05, **p < 0.01 (two-tailed unpaired t-test). Scale bars, 10 μm.
We next examined the mechanism of SAP-mediated inhibition of HMGB1 and LL37 function. As SAP has a wide spectrum of ligands, including alarmins, C1q, and many other endogenous proteins (25), we determined whether hSAP could directly bind to the two proteins. We used HMGB1- and LL37-containing human serum to detect the association of HMGB1, LL37, and SAP with an immunoprecipitation analysis, using C1q as a positive control protein. We did not detect HMGB1 or LL37 binding with immunoprecipitated SAP. This result indicates that hSAP does not bind HMGB1 or LL37 directly (Fig. 3D).

We next hypothesized that SAP in complex with DNA might competitively inhibit the function of other DNA-binding proteins. To investigate this hypothesis, we incubated biotin-labeled LL37 with ISD in the presence of increasing amounts of hSAP and then separated the complexes on native PAGE gels. We found that free biotin-labeled LL37 was significantly augmented with increased hSAP, which indicates that LL37 was released from the complexes in the presence of hSAP (Fig. 3D). We also compared the binding of immobilized HMGB1 or LL37 to biotin-labeled ISD in the presence of hSAP or mSAP with a competitive ELISA assay. Consistent with the gel-retention results, we found that the amount of bound DNA significantly decreased when the concentration of hSAP was increased; mSAP and CHPHC-treated hSAP did not have this effect (Fig. 3E). These results indicate that the DNA-binding ability of both HMGB1 and LL37 can be inhibited by hSAP under physiological conditions.

hSAP changes DNA uptake manner from macropinocytosis to endocytosis

To further study the mechanism of SAP-mediated inhibition to innate immune activation, we investigated the interactions between hSAP–DNA complex and macrophages. Previous studies have shown that hSAP can bind CD14+ PBMCs and opsonize the ligand zymosan for phagocytosis (24–26, 34). Therefore, we explored whether the hSAP in DNA–protein complexes regulates the uptake of DNA by macrophages. The DNA uptake efficiency was determined by flow cytometry using fluorescently labeled DNA. We also used HMGB1 and LL37 to simulate physiological transfection agents based on the previous reports. However, the intracellular DNA did not increase in the presence of hSAP (data not shown). As endocytic naked DNA is always degraded rapidly by endosomal nucleases, we used chloroquine to prevent endosome acidification. We then observed that hSAP significantly increased the intracellular fluorescence rates of MDMs (Fig. 4A), suggesting that hSAP facilitates DNA uptake by macrophages but degraded in endosome subsequently. These findings were further confirmed using confocal microscopy (Fig. 4C), and we also found that the hSAP–DNA complex was well correlated with the fluorescence rate by Western blotting. The fluorescence-positive cell rates are shown (mean values and SD). *p < 0.05, **p < 0.01 (two-tailed unpaired t test).

FIGURE 5. hSAP facilitates plasmid DNA endocytosis via FcRs. A, FITC-labeled DNA was coincubated with hSAP and then added to THP-1 MDMs in the presence of the specific inhibitors for AGER, FcγRs, and scavenger receptors. The intracellular DNA was determined with flow cytometry. The fluorescence-positive cell rates are shown as mean and SD based on three independent experiments. *p < 0.05, **p < 0.01 (two-tailed unpaired t test). B, hSAP, DNA, and their complexes were incubated with MDMs for 10 min, and Syk activation was determined by assessing the phospho-Syk (p-Syk,Tyr525/526) level by Western blotting. C, HEK293 cells transfected with different receptors were incubated with FITC-labeled DNA in the presence of hSAP or CRP, and the plasmid pcDNA3.1(–)-flag was used as a negative control (NC). The fluorescence-positive cell rates are shown (mean values and SD). *p < 0.05, **p < 0.01 (two-tailed unpaired t test). All samples were tested in duplicate.

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found that uptake partly requires an intact actin cytoskeleton and PI3K activation, suggesting that receptor-mediated endocytosis plays a role. For LL37 preincubated DNA, methyl-β-cyclodextrin, amiloride, and latrunculin A treatments all resulted in significant decrease of DNA uptake (Fig. 4E), which is consistent with the previous report that LL37 facilitates DNA uptake via macro-pinocytosis (31). All those results indicated that the DNA uptake manner of MDMs changed to endocytosis in the presence of SAP.

**hSAP facilitates DNA endocytosis mainly in an FcγR-dependent manner**

To further determine the dependent receptors of SAP-mediated DNA uptake, we used IVIg (7), polyG (36), sRAGE (37), and piceatannol (7) to inhibit FcRs, scavenger receptors, AGER, and the FcR adaptor Syk, respectively (Fig. 5A). In the absence of SAP, only cells treated with sRAGE or polyG showed a significant reduction of native DNA uptake, and this result is consistent with previous reports on the role of the HMGB1–RAGE interaction (38) and scavenger receptors in DNA uptake (39). However, in the presence of SAP, the DNA uptake increase could only be inhibited by IVIg and piceatannol, which suggests that FcRs play a key role in SAP-mediated DNA uptake (Fig. 5A). The data also indicate that scavenger receptors and AGER-mediated DNA uptake is inhibited in the presence of SAP. We also examined whether activation of the Syk signaling pathways through the FcRs is affected by the hSAP–DNA complex. We found that hSAP alone induced significant Syk activation, and at the same time, both the hSAP–CpG-B and hSAP–ISD complexes have a weaker but definite ability to activate Syk (Fig. 5B).

To further identify the dependent receptors, we cloned a group of receptors including FcyRIa, FcyRIIa, FcyRIIb, FcyRIIIa, SRA, and AGER, and transfected them into HEK293 cells. Those receptor-transfected cell models were used to study the hSAP-DNA uptake efficiencies with CRP, another short pentraxin without DNA-binding ability, as a control. Our results indicated that only the cells overexpressing FcyRIa and FcyRIIIa had an increased ability to uptake the hSAP–DNA complex (Fig. 5C), which also suggests that the FcγRs play a role in SAP-mediated DNA uptake.

**hSAP suppression promotes innate immune responses to DNA under physiological conditions**

For ethical reasons, it is still not possible to further determine the role of hSAP in the regulation of immune responses to DNA vaccine in clinical investigations. However, in this study, we further investigated the innate immune responses to DNA under a simulated natural environment in human body. We added human serum from healthy individuals into the MDM culture, and we found that DNA-induced immune response, including the CpG-B–induced CCL5, TNFA, and IL-6 mRNA production or the ISD-induced IFN-B1, IFN-A4, and IL-6 mRNA production was significantly inhibited in the presence of human serum. However, with increasing doses of CHPHC, we found a significant increase

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**FIGURE 6.** hSAP in negative regulation of innate immune responses. A, THP-1 MDMs were stimulated with CpG-B or ISD with 10% human serum in the presence or absence of the SAP inhibitor CHPHC. Cytokine mRNA expression was determined by quantitative real-time RT-PCR. The relative expression levels were assayed using the $2^{-\Delta\Delta C_t}$ method and are shown as mean values and SD based on three independent experiments. B, Mice (five per group) were i.v. injected with B-DNA in the presence or absence of the CHPHC. The serum cytokines levels were determined by ELISA and are shown as mean values and SD based on three independent experiments. **p < 0.01, two-tailed unpaired t test, significant differences between groups. ND, not detected.
of cytokine mRNA after DNA stimulation (Fig. 6A). These results indicate that some agents in natural serum inhibit the DNA-induced immune response, and that SAP-specific inhibition may break the physiological barrier to induce immune activation, further suggesting the role of SAP in the negative regulation of DNA-sensing systems.

We also investigated the effects of SAP-specific inhibition on the innate immune activation by B-DNA in vivo in the hSAP Tg mice. We found that i.v. injection of B-DNA induced a significant increase in serum IL-6 and TNF-α levels in the WT mice, but the response was much weaker in the hSAP Tg mice. However, the coadministration of CHPHC partly recovered the B-DNA-induced serum cytokines production in the hSAP Tg mice (Fig. 6B), and this result further indicates that hSAP inhibits innate immune recognition to B-DNA in vivo. Based on the previous results, we hypothesized that hSAP regulates DNA-induced immune responses by suppressing the functions of other extracellular DNA-binding proteins. A schematic view of this hypothesis is shown in Fig. 7.

Discussion

The drive to improve DNA vaccine function is fueled by the consensus that DNA vaccines may be immunologically benign; that is, they are simply not carrying enough of the signals necessary to trigger a strong innate immune response (40). Thus, there is an urgent need for more robust and universally applicable adjuvant strategies to facilitate immune activation during DNA vaccination. However, despite the gradually uncovered mechanisms by which extracellular dsDNA activates innate immune responses, the agents that negatively regulate extracellular DNA sensing remain unclear. In this article, we have provided evidence that hSAP can universally inhibit the activation of various DNA sensors through a mechanism involving competitive inhibition of the DNA sentinels HMGB1 and LL37. As hSAP is proved to be the major extracellular DNA-binding protein naturally circulating in serum and presenting in normal extracellular matrix (22, 41, 42), we further suggest that hSAP may play a key role in the negative regulation of the innate immune response to DNA vaccines under physiological conditions.

It has been shown that free DNA and DNA sentinels, such as HMGB1 and LL37, normally exist in the extracellular environment and greatly increase during infection and tissue damage (7, 43). Because SAP is a natural constitutive component in human serum, we hypothesize that SAP can “arrest” those extracellular DNAs to avoid their binding with DNA sentinels and then forbid them from “passing” the barrier to activate innate immunity. Only when extracellular DNA or DNA sentinels greatly increase and exceed the threshold of SAP-binding ability can DNA be captured by HMGB1 and LL37 and engulfed to activate DNA sensors. The innate immune response may be subsequently initiated. This hypothesis may partly explain the previous findings that multiple high doses of DNA vaccine are always necessary to increase immune efficacy in many human applications (44). In this regard, we suggest that hSAP may play a key role in the regulation of the immune responses to DNA vaccine under physiological conditions.

For the mechanism of innate immune suppression induced by SAP, we compared the different uptake manners between SAP and other DNA-binding proteins. It is shown that HMGB1 and LL37 facilitate DNA uptake mainly though a macropinocytosis manner, which increases cytosolic DNA concentrations with a “leakage” mechanism (8, 31) and then facilitates DNA sensor activation. However, in our findings, SAP competitively inhibits the function of HMGB1 and LL37, and facilitates DNA’s uptake via endosomal pathways leading to degradation. We therefore suggest that it may also be a potential mechanism of SAP-mediated inhibition to innate immune recognition of DNA.

Opsonization by SAP is controversial in the literature. Many studies have shown that SAP opsonizes apoptotic cells and zymosan through FcγRs (24, 26, 45), but in contrast, SAP has been shown to be a potent antiopsonin (46) in many other reports. Specifically with respect to genomic DNA, SAP has been reported to protect chromatin against degradation (47). In this study, we observed the engulfment of SAP-opsonized B-DNA by human MDMs and found that the SAP–DNA complex localized in the early endosome. However, the amounts of engulfed DNA in MDMs did not significantly change in the presence or absence of hSAP, which indicates that SAP-mediated endocytosis is inefficient in DNA uptake. For CpG-B DNA, we even found that hSAP inhibited their uptake by MDMs. We therefore assume that most of the SAP–DNA complex may not be phagocytosed immediately and may be gradually cleared by macrophages for a long period. Because rapid engulfment of a large amount of DNA may induce unexpected overactivation of the innate immune response, the “slow-clearance” mechanism of the SAP–DNA complex may also be helpful for maintaining the balance of DNA-related immunity during infection and tissue damage.

There is another interesting problem to be discussed. Although we observed the engulfment of SAP-opsonized B-DNA by human MDMs and found that the SAP–DNA complex localized in the early endosome, these DNAs did not activate endosomal DNA sensors, such as TLR9. Based on previous CpG-A data, multivalent higher order structures of DNA may be necessary for TLR9 cross-linking and activation (48). We believe that the SAP–DNA complex may alter the formation of DNA higher order structures and affect the TLR9–DNA interaction in the early endosome because SAP is strongly resistant to protease degradation. Whether
SAP inhibits DNA from assembling into larger, multivalent complexes remains to be determined. Previous studies have shown that aggregated SAP alone can stimulate the production of IL-10, IL-8, and IL-6 by CD14+ monocytes via the FcγR-dependent pathway. However, in this study, although we also confirmed that hSAP facilitates DNA endocytosis via FcRs, we found that hSAP inhibits IL-6 production but stimulates IL-10 production when it complexes with DNA. The differences may be related to the structure alternation of the SAP–DNA complex. Aggregated SAP without ligands has been shown to have little binding activity for FcγRIIB, the human inhibitory receptor, but has the greatest affinity for the activating receptors FcγRIIA and FcγRIIb (26, 45). However, the preference of SAP may be altered after binding with DNA. SAP shows an impaired ability to activate Syk in the presence of DNA ligands, which further suggests the difference between the SAP–DNA complex and SAP alone. The problem needs further study to examine alterations in the molecular structure.

The different activity of SAP between human and mouse has long been known, but it is still interesting to imagine that it contributes to the species-specific efficiency of DNA vaccines. In previous studies, SAP-deficient mice developed higher titers of autoantibodies against nuclear debris than WT mice; the authors proposed that SAP, by stabilizing and transporting chromatin to the liver for catabolism, may prevent the formation of Abs against DNA (47). However, further studies indicated that most of the autoimmunity originally reported in SAP-knockout mice was due to genetic effects of the mouse strain background and not SAP deficiency (23, 49). As such, the role of mSAP in DNA clearance is ambiguous. In this study, we found that mSAP has no significant function in the innate immune response to DNA, which further suggests that hSAP has a species-specific ability to regulate DNA immunity. These findings may have implications for understanding the evolution of the innate immune system.

As Wolfgang W. Leitner previously claimed (40), the honey-moon period that vaccinologists had with the new technology of DNA immunization is over with the realization that DNA vaccines were not as effective in human clinical trials as they were in mouse-based studies. Finding some ways to overcome the species-specific obstacles is therefore an urgent problem in current vaccine studies. Although various attempts have been made to find adjuvant strategies that will enhance the immunogenicity of plasmid DNA vaccines, and some of them, such as the electroporation, improved chemical adjuvants, which should be more convenient, and painless, may allow plasmid DNA to become an immunization of choice. Although various attempts have been made to find adjuvants that will enhance the immunogenicity of plasmid DNA vaccines, and some of them, such as the electroporation, improved chemical adjuvants, which should be more convenient, and painless, may allow plasmid DNA to become an immunization of choice.

Disclosures
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References

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