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Hydrogen peroxide-inactivated bacteria induces potent humoral and cellular immune responses and releases nucleic acids

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ABSTRACT

The problem of nosocomial infection is seriously escalating. Bacterial vaccines are indispensable for preventing infections caused by multi-drug resistant organisms. Some researchers have put forward the use of hydrogen peroxide (H_2O_2) as a new technology platform for virus deactivation. This deactivated virus can induce the number of $CD8^+$ T lymphocytes, which can enhance antiviral responses. Although, H_2O_2 treatment has been rarely reported on the exploration of bacterial deactivation, H_2O_2 deactivation of whole-cell bacteria could be a potential novel approach for bacterial vaccine development. Here we present a strategy for H_2O_2-deactivated bacterial whole-cell vaccines, for two major pathgens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The proactive effects of vaccination were assessed in vitro and in vivo.

H_2O_2-deactivation of bacterial vaccines retains more complete epitopes and exhibits lower toxicity, as compared to formaldehyde, a conventional deactivator that was investigated in this study. Furthermore, H_2O_2-deactivated bacterial vaccines induce anti-infection responses through enhancement of humoral immunity and cellular immunity. Vaccination with H_2O_2-deactivated whole-cell bacteria in mice mainly elicits whole-cell specific antibody titers and balances the IgG2a and IgG1 response, predominantly with IgG3 induction at the later stages, meanwhile provides opsonic protection against challenge with pathogens. Finally, H_2O_2 deactivation of bacteria has been found to cause the release of bacterial DNA which is followed by NF-κB activation. These findings demonstrate that the deactivation of whole-cell bacteria with H_2O_2 is potentially advantageous for immune responses. Considering the prevention of drug-resistant infections, this deactivation method could be simultaneously applied as an innovative strategy for bacterial vaccine development.

1. Introduction

According to the collected Data from the Disease Control and Prevention (CDC) centers, approximately 23,000 death per year is occurred in the United States due to drug-resistant bacterial infections. Infection rate of *Pseudomonas aeruginosa* (*P. aeruginosa*) is higher in the burn, cancer, and immunocompromised patients. A number of *P. aeruginosa* virulence factors and delivery systems have been tested in Phase I–III clinical trials, including vaccines against bacterial components (e.g. lipopolysaccharides, flagella) [1]. Variety of effective vaccines against *P. aeruginosa* are currently available, but *P. aeruginosa* infections due to its antibiotic resistance are increasing. In order to improve the presentation of multiple antigens to the immune system, we would focus on Whole-cell killed or live-attenuated vaccines as stated in the literature [2–4]. An oral, whole-cell *P. aeruginosa* vaccine (Pseudostat) administered to 30 healthy volunteers was associated with significant production of specific IgA [2]. *Staphylococcus aureus* (*S. aureus*) is the other bacteria that represents multiple drug-resistant strains, appears in a very broad range of

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diseases. Attempts to prevent *S. aureus* infection with vaccine have failed for numerous reasons, including incorrect selection of antigen, which caused weak bactericidal activity of the antibodies in neutrophils, and opsonization [3]. It is therefore, in order to counteract with these insurmountable bacteria, some new antibacterial agents have been developed. Antimicrobial activity of DP7 (VQWRIRVAVIRK), as a novel antimicrobial peptide was tested in a *S. aureus* infection murine model [5]. In addition, an oil-in-water nanoemulsion adjuvant vaccine containing an MRSA recombinant protein antigen is a small molecular drug that could improve the specific immune responses of IgG1, IgG2a, IgG2b, IgA in the serum [6]. In addition, different modes of inactivation of *P. aeruginosa* and *S. aureus* were found for the same plasma source depending on the solution and the plasma feed gas [7].

An agent associated with the vaccine production, formaldehyde, is an effective deactivation, however, it affects the integrity of epitope. In order to solve this problem, researchers have proposed using hydrogen peroxide (H_2O_2) for viral deactivation [8,9]. It was found that this method retained the immunogenicity of virus RNA or DNA and induced $CD8^+$T cell-mediated immunity [10]. On the other hand, the H_2O_2-lymphocytic choriomeningitis virus (LCMV) elicits neutralizing of antibody responses simultaneously. At the present, there are few studies reporting the deactivation of bacteria by H_2O_2. Nebulization of hydrogen peroxide (H_2O_2) in low concentrations to the lower pathogenicity of APEC was tested in the presence of chickens [11]. Likewise, it has been used to detoxify pertussis toxin [12].

The form of vaccine antigen plays an important role in dendritic cells, B cells, and T cells in the interaction germinal center that affects the duration and intensity of protective immunity [4]. Oxidation-specific epitopes (OSEs) that are sensed by cellular pattern recognition receptors (PRRs) are recognized by a variety of cell surface receptors [13]. In this investigation, it is intended to employ this new type of deactivation strategy in bacterial vaccine application, and to observe the anti-infection response and effectiveness of H_2O_2-deactivated bacterial vaccine, especially the synergistic reaction of humoral and cellular immune responses during immunization process. Furthermore, the mechanism of immune enhancement is analyzed.

2. Materials and methods

2.1. Mice and cell lines

Six- to eight-week-old female Balb/c, were purchased from Beijing Laboratory vital river Animal Co. Ltd. All mice were maintained in the specific pathogen-free facility. All experimental Protocols and animal care were reviewed and approved in accordance with the regulations of the Animal Care and Use Committee of Sichuan University. **Cell culture**, RAW264.7 cells (mouse macrophage cell line) were grown in culture RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco). Caco-2 cells were grown in culture Dulbecco's Modified Eagle's Medium (DMEM) with 20% FBS. The number of RAW264.7 and Caco-2 cells passages was three to five, with an average of two days.

2.2. Bacteria

Bacterial strains of *Pseudomonas aeruginosa* (PAO1), *S. aureus* (ATCC 33591), and *E. coli* (ATCC 25922) were purchased from the American Type Culture Collection (Rockville, MD). RE88 was an attenuated engineered *Salmonella* strain with deletion of *Dam* and *aroA*. It was a kind gift from Rong Xiang and colleague (The Scripps Research Institute, La Jolla, CA, Nankai University School of Medicine, Tianjin, China).

2.3. Preparation of rabbit anti-PAO1 serum

When PAO1 were in log phase, prepared the bacteria in the 2 mM PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO4 0.44 g and KH_2PO4 0.24 g in 1000 ml distilled water, PH = 7.4), the concentration was 10^7 CFU/ml. Subcutaneous multi-points injection with 1000 ml PAO1 solution was performed in the back of New Zealand rabbits (female each with about 2.0 kg), for each site we used a suspension volume of 150–200 μl and each point contained about 2×10^6 CFU organisms. The immunization schemes were as follows: one administration on day 0, day 14, day 28 and day 56, a total of 4 times, while the vaccine mixture consisted of bacteria solution and adjuvant, complete Freund adjuvant was considered for the first time and incomplete Freund adjuvant for the other three times. 100 ml venous blood was from the heart one week after the last immunization. We collected the venous blood from the heart and incubated in 37 °C for 1–2 h, the incubated serum was then centrifuged in 3000 rpm for 10 min and packed under −20 °C conditions.

2.4. Preparation of bacterial strains on vaccination

Overnight-shook bacteria on LB were collected and washed with sterile PBS. Bacteria were deactivated in 37 °C with H_2O_2 or formaldehyde in different concentrations. After reaction, the prepared bacterial strains was washed and suspended to adjusted to 10^8 CFUs (colonies forming units) per milliliter of PBS. Ultraviolet absorption spectrophotometry was used to measure OD value for bacterial concentration. The 1.0 OD value at 600 nm was equal to about 10^8 CFU bacteria per milliliter solution.

2.5. Immunization

For immunization, the bacterial suspension was inactivated in 3% H_2O_2 or HCHO solution without any adjuvants. The proportion of H_2O_2 or HCHO is given as weight per volume (wt/vol). Six to eight-week-old female Balb/c mice were vaccinated with 3×10^7 CFU PAO1 or 3×10^8 CFU 33591 strains, with dose of LD50 (Lethal Dose, 50%), and boosted with the same vaccine at 14 d and 28 d after the primary vaccination. The negative control group was defined as the mice injected with PBS buffer.

2.6. Challenge and euthanization

The challenge was performed with live bacteria in 10 doses of LD50 per mouse, the attack dose of the bacteria were 3×10^8 CFU per mouse for PAO1, and 3×10^9 CFU per mouse for ATCC33591. PAO1 and 33591 suspensions were collected at the logarithmic phase of the culture growth and washed in PBS, 500 μl bacterial solution was injected i.p. eight weeks after the first immunization. In histological analysis, mice were sacrificed eight weeks after the first immunization. We took mouse orbital vein blood and separated the serum to test the antibody titers or others. The heart, liver, kidney were removed in PBS containing 4% paraformaldehyde at room temperature followed by placing in concentration gradient ethanol to dehydration prior to paraffin embedding. Tissues were stained with hematoxylin and eosin after section.

2.7. Enzyme linked immunosorbent assay (ELISA)

Immunoabsorbent plates (96-well, NUNC) were coated with 10^6 bacteria/well in 20 mM sodium bicarbonate (pH = 9.6) overnight at 4 °C. The wells were washed with 0.05% PBST (0.05% Tween20 in PBS), blocked with 5% non-fat milk in PBST for 1 h and incubated with the double diluted sera for 1–2 h. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (ZSGB-BIO, China), IgG1, IgG2a, IgG3, IgA or IgM (Southern Biotech) were added and incubated for 1 h in 37 °C, the dilution ratio of secondary antibodies was referred to the provided specifications by the supplier. After washing, SureBlue™ TMB Microwell Peroxidase Substrate (KPL, MD) was added for 10 min under darkness condition and then stopped with 1 M H_2SO_4. Plates were measured by the optical density at 450 nm using a Multiskan Spectrophotometer (Thermo Fisher Scientific, Yokohama, Japan). For the anti-PAO1

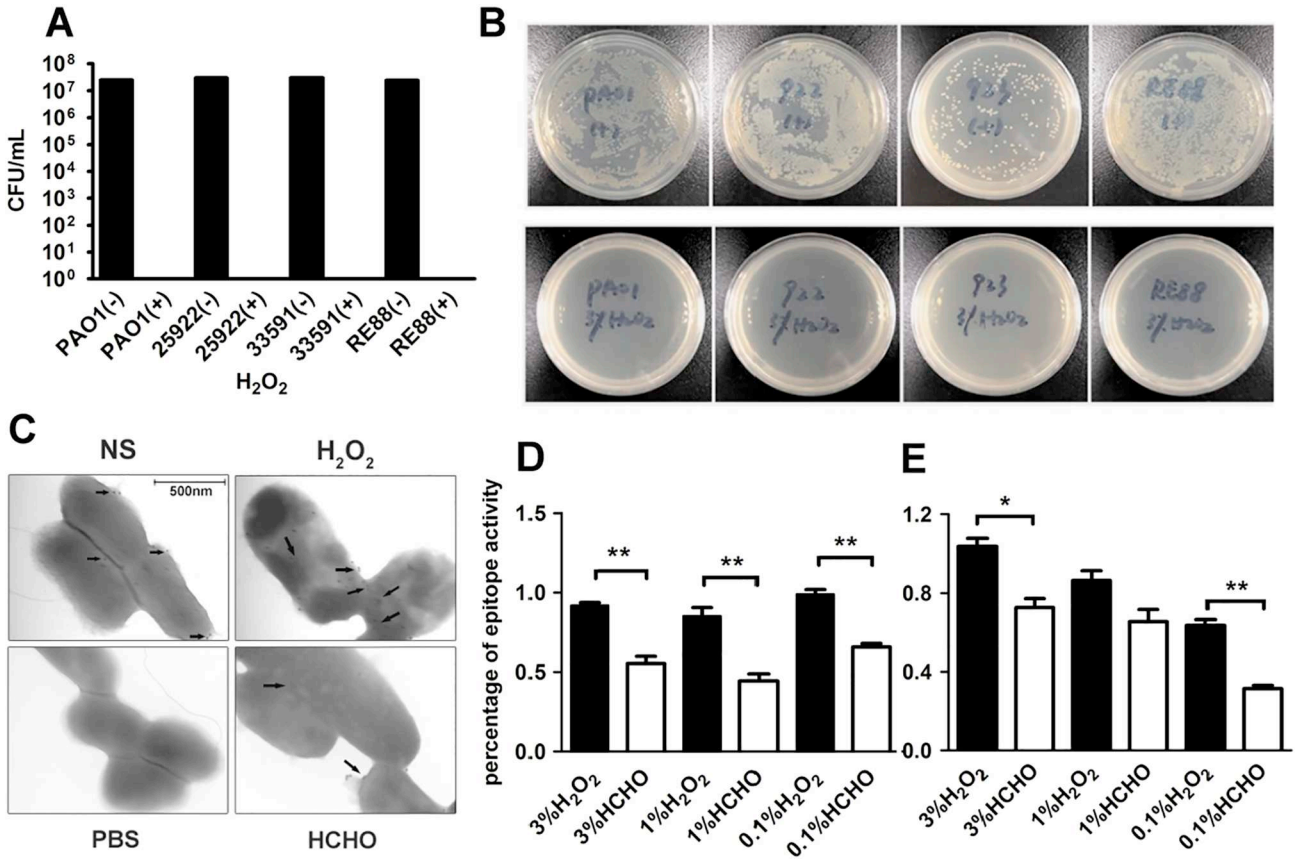

Fig. 1. The effect and advantage of hydrogen peroxide (H_2O_2) in killing bacteria. PAO1, ATCC25922, ATCC25923, RE88 were treated with 3% H_2O_2 at 37 °C after 2 h and then cultured on LB plates to allow bacterial growth was observed (A,B). Compared with the same concentration of formaldehyde which was represented by HCHO in this figure, the damage degree of PAO1 surface antigen epitope with anti PAO1 rabbit serum antibody was detected after the treatment (C), after incubation with PAO1 rabbit serum overnight, the deactivated bacteria were incubated with diluted colloidal gold labeled Goat anti rabbit IgG (Boster, GA1013) as displayed by black arrow in part C. Colloidal gold dilution liquid was PBS and 1% reagent grade BSA was added according to the manufacturer's instructions. Simultaneous processing time for detection of 3% H_2O_2 by enzyme with linked immunosorbent assay (ELISA) was 2 h (D) or 24 h (E) for epitope activity, the percentage showed an absorption ratio of pre-deactivation group and treated groups. Data were showed as mean ± SEM of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. the 3%HCHO, 1%HCHO, 0.1%HCHO groups.

epitopes analysis in Fig. 1, plates were coated in the treated whole-cell PAO1 and rabbit anti-serum was used as the first-antibody.

2.8. Detection of flow cytometry

Mice were sacrificed for evaluation of their spleens while non-infected mice were considered as the negative control. Splenocytes were prepared in advance as described in the reference [18] in 6-well plates at 2×10^7 cells per well overnight. The isolated spleen lymphocytes were stimulated with 50 μg/ml bacterial lysate for 24 h, then washed with PBS and diluted to 10^7 cell/ml [19]. Cells were incubated at 4 °C for 30 min after adding a fluorescent labeled antibody, FITC-Rat-anti-mouse-CD4, PE-Rat-anti-mouse-CD44 and APC-Rat-anti-mouse-CD62L (BD Biosciences), fixed with 2% poly formaldehyde. After saponin drilling, cells were stained with intracellular antibody, APC-Rat-anti-mouse-IFN-γ and APC-Rat-anti-mouse-IL-4. Samples were analyzed immediately using a FACS Calibur and BD CellQuest TM Pro software.

2.9. Western blotting

RAW264.7 is a murine leukemia cell line with similar physiological characteristics to those of antigen-presenting cells. After stimulation with bacterial solution for 5, 10, 15, 30, 45, 60 min, the cells were lysed by RIPA (Beyotime, China) which had been supplemented with a protease inhibitor cocktail (Roche). We collected the extracted protein from RAW264.7. Samples were loaded and run on 10% SDS-PAGE gel. The proteins were transferred to PVDF membrane and then blocked with 5% non-fat milk in TBST (0.1% Tween20, 150 mM sodium chloride, 50 mM Tris-HCl, pH = 7.4). First antibodies, such as GAPDH (14C10) rabbit mAb, NF-kappaB p65 antibody, Phospho-NF-kappaBp65 (Ser536), were purchased in *cell signaling technology*, secondary antibody was HRP-conjugated with anti-rabbit antibody. The membrane was washed in TBST and developed with the ECL detection kit (Amersham Pharmacia Biotech).

2.10. Opsonophagocytic assays

The bacterial clearance rate of the spleen was conducted using the similar method that described by other researchers [2,14–16]. Four days after the challenge, mice serum was collected. It was observed that the reaction system volume was 400 μl while 2.5% fetal bovine serum provided the source of complement. The concentration of human neutrophils was 5×10^6 cells/ml, respectively at which the volume was 50 μl. 200 μl tested serum was diluted in appropriate proportion and 5×10^6 CFU PAO1 was added as well. Each solution was diluted with DMEM medium. The mixture was incubated for 90 min at 37 °C, then diluted the solution to 1/10,000 and plated on LB medium for CFU counting. Neutrophils were isolated from human venous blood (Axis-Shield, polymorphprep), whereas negative control sera were from unimmunized mice.

2.11. Anti-toxin test of whole bacteria neutralization

Caco-2 were collected in 96-well plates with concentration of 5×10^4cell/well which had been incubated for 4–6 h (10%FBS, 37 °C, 5% CO_2). Anti-serum of deactivated-PAO1 immunized mouse and a certain number of alive or inactive bacteria were added. Bacterial suspension was diluted to the expected concentration in DMEM media which had been co-incubated for another 4 h. The mixture was then treated with CCK-8 for 1 h while alive bacteria was used as toxin control and absorbance was measured at 570 nm. Neutralization is calculated using the following equation; %neutralization = (Sample OD − toxin control OD) / (cell control OD − toxin control OD) ∗ 100.

2.12. Data analysis

Statistical analysis was performed with ONE-WAY ANOVA by using SPSS17.0 statistical relevance between groups at which P-value below than the 0.05 was considered as significant level. After comparing the ANOVA, we chose the scheffe method that focuses on difference between the test groups.

3. Results

3.1. The process of bacteria inactivation by H_2O_2

To examine the inactivation effect of H_2O_2, four different strains of bacteria were selected for this study, *S. aureus* (ATCC33591), *P. aeruginosa* (PAO1), *E. coli* (ATCC25923), and *Salmonella typhi* (RE88), RE88 is commonly used as a bacterial carrier with double deletion of *Salmonella*. After inactivation, the bacterial suspension was diluted and cultured a certain volume in liquid nutritious broth or on LB plates. Un-inactivated bacteria were used as a control. After incubating at 37 °C for 16–18 h in a CO_2 incubator, the liquid culture of the inactivated strain was clear, and no colonies were observed on the plates, indicating that the medium had been effectively sterilized. Notably, 3% H_2O_2 in PBS effectively suppressed the growth of all four bacterial strains (Fig. 1A and B). It was also confirmed that no infectious bacteria were visually detected in solution on plates after 2 h of inactivation with formaldehyde at concentration as low as 0.1%. The same concentration of hydrogen peroxide had a comparable inactivation effect (Fig. S1). The activity of antigen epitopes on H_2O_2-inactivated bacteria or formaldehyde-deactivated bacteria was tested using ELISA (Fig. 1D and E). We found that the deactivation time required by H_2O_2 treatment was lower than that required by formaldehyde. Conversely, it was observed that H_2O_2 killed the bacteria while it was fully bound to the mixed anti-PAO1 IgG, which is possibly due to its immunogenic integrity or due to the higher exposure of proteins on its surface. Furthermore, through microscopic observations, we observed that more gold particles were adhered to the surface after H_2O_2 treatment, while fewer particles were observed upon formaldehyde treatment, suggesting that deactivation of bacteria with formaldehyde eliminated some of the surface epitopes (Fig. 1C). Thus, the maintenance of plentiful surface antigens by H_2O_2 deactivation stimulated a stronger immune response for more efficient immunogenicity.

3.2. In vivo anti-infection effects of H_2O_2-inactivated bacteria vaccines

Bacteria can express a variety of surface antigens, such as LPS, exotoxin, and lipopolysaccharide, which could be used as a whole-cell vaccine. Therefore, we assessed the in vivo effects of H_2O_2-bacteria in mice. After three immunization steps of BALB/c mice with inactivated bacteria, we challenged the mice with bacteria at 10LD50 dose (Fig. 2A). First, the vital organs was explored after the immunization. As shown in Fig. 2B, no obvious pathological changes were observed in the H_2O_2-group, while some disturbances appeared in the formaldehyde group. Likewise, there were a higher number of vacuole-like cells in the liver of mice treated with formaldehyde, demonstrating that the toxicity of H_2O_2 was weaker than formaldehyde (Fig. 2B). Survival analysis revealed that the survival rate of the animals in the H_2O_2-deactivated bacterial vaccine group was significantly higher than those in the formaldehyde group (Fig. 2C and D). The protective effect of H_2O_2 positively correlated with the dose of the H_2O_2-deactivated bacterial vaccine (Fig. 2E). These results reveal that H_2O_2-deactivated bacterial vaccine has substantial antibacterial effect on prophylactic immunization.

3.3. H_2O_2-inactivated bacteria induce the balanced IgG response

Based on the results presented above, the advantage of H_2O_2 over formaldehyde deactivation was clearly demonstrated. To investigate the effect of H_2O_2 deactivation, we performed serum immunoglobulin (Ig) titer testing to evaluate the host responses. For both *P. aeruginosa* or *S. aureus*, the titer of H_2O_2-deactivated group was significantly higher than that of the formaldehyde-deactivated group (Fig. 3A and B). The IgG titer was the highest 56 days after first immunization with formaldehyde-deactivated bacteria, while the IgG titer in the H_2O_2 group was remarkably higher (Fig. 3C and D).

In the detection assay of different IgG subtypes, there was a little difference between the two types of bacteria strains. In the H_2O_2-PAO1 group, titers of IgG1 and IgG2a were distributed relatively uniformly. In the H_2O_2-33591 group, the proportion of IgG1 decreased (Fig. 3E and F, Fig. S4). However, in both bacterial groups, the ratio of IgG2a/IgG1 in the H_2O_2 group was higher than that in the formaldehyde group, which was close to one (Table S1). This would affirm that H_2O_2 deactivated bacteria probably balanced the IgG2a and IgG1 immune responses. In contrast, formaldehyde-deactivated bacteria mainly induced the IgG1 immune response (the ratio of IgG2a/IgG1 was always < 0.5). Compared to conventional bacterial vaccines that are based on bacterial components or immune complexes [20,21], this novel H_2O_2-deactivated bacterial vaccine promoted an alternative subtype immune response. The levels of IgG3 were more prominent until the 56th day (Fig. 3G and H) and the titer was maintained high than 10^4. This observation was similar in both types of bacteria. The H_2O_2-PAO1 group showed a rather high IgM titer at the early stage. Although the IgA titer is closely related to mucosal immunity, it was higher in the H_2O_2 treated group compared to the formaldehyde treated group, while the titer was far lower than the IgM titer in the H_2O_2 treated group (Fig. S2).

According to the literature, which includes studies on subunit or DNA vaccines [22–25], in splenic active or proliferative T cells, higher levels of IFN-γ secretion reflect resistance to pathogen infection. In this study, we found that H_2O_2-deactivated bacterial vaccines could seemingly stimulate cellular immunity, as activation of T cells and secretion of IFN-γ was observed in H_2O_2-deactivated bacterial vaccine immunized mice. Activation of T lymphocytes in the spleen was slightly higher in the H_2O_2 group in the later stage after immunization (Fig. D), showing that H_2O_2-deactivated bacterial vaccines positively regulated the immune response. 35 days after immunization, the observed rise in $CD4^+IL\text{-}4^+$ T cells of H_2O_2 group was also greater than that of HCHO group, while $CD4^+IL\text{-}4^+$ T cells increased in HCHO vaccination early stage slightly. (Fig. 4C). The IgG isotype-dominated response has been widely reported depending on different $CD4^+$ T cell subsets, the presence of both IFN-γ and IL-4 may promote the simultaneous production of IgG2a and IgG1. It reflected in H_2O_2 group which far generated more IgG2a in serum associated with $CD4^+IFN\text{-}\gamma^+$ T cell (Fig. S4). Then the ability of memory $CD4^+$ T cells was investigated after PAO1 infection. Memory $CD4^+$T cells are crucial for T cell immunity against infection, but how they contribute to protection is not well understood. We found that $CD44^+CD62L^+$ T cells in the spleen enhanced expression in the H_2O_2-PAO1 treated group almost three months after immunization, compared to HCHO-PAO1 treated group (Fig. 4A and B), suggest that memory cells could have key functions at whole stage of infection.

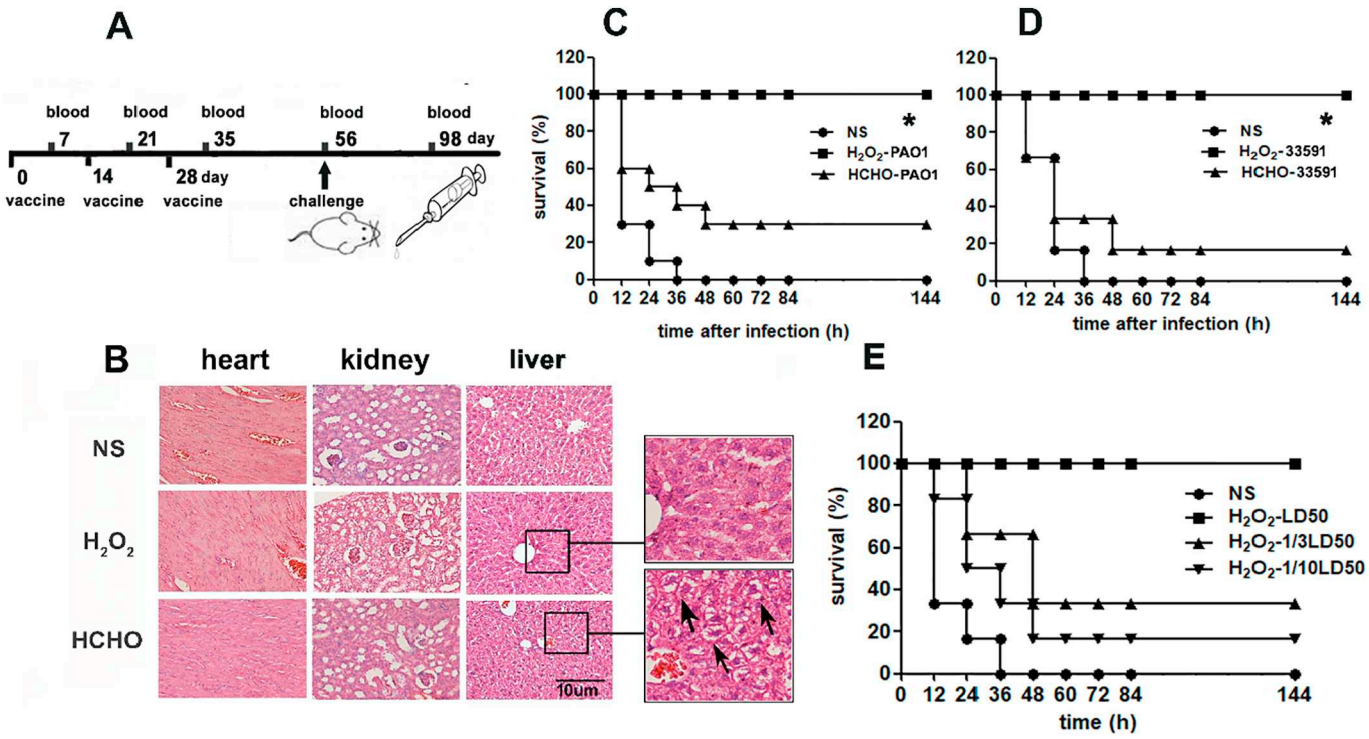

Fig. 2. Advantages of H_2O_2-deactivated bacterial vaccine in vivo. The process of vaccination is displayed (A), adult Balb/c female mice were administered in two kinds of deactivated whole-cell bacteria in doses of LD50 (LD50 of PAO1 was 3×10^7 CFU per mouse, LD50 of ATCC33591 was 3×10^8 CFU per mouse) and sacrificed eight weeks after the first immunization. Then the main organs were removed in PBS containing 4% paraformaldehyde and hematoxylin and eosin (HE) staining was performed (B), mice were attacked in dose of 10LD50 and variation of survival mice is demonstrated observation of survival mice varies with time (C, D), and survival rate of different doses in H_2O_2-PAO1 (E). Data were mean ± SEM of three independent experiments, 10 mice were used in the survival rate assay (n = 10), and 3 mice were used in the HE staining assays (n = 3). *$P < 0.05$ as compared with the HCHO-PAO1, HCHO-33591 groups.

3.4. Higher opsonization of H_2O_2-inactivated bacterial vaccine than HCHO-inactivated bacterial vaccine

Binding of neutralizing antibodies is a characteristic of bacterial immune response, which includes opsonin phagocytosis and secretion of anti-bacterial toxins [16,26]. Based on the serum opsono-cytophagic test, over 10% of the bacterial death rate was achieved in all the dilutions tested in the H_2O_2-33591 group. The death rate induced by 1/18 diluted serum was > 40% (Fig. 4E). Deactivation of 33591 by formaldehyde revealed opsonic killing activity; however, the killing efficiency was not as high as that of the H_2O_2 group. The EC_{50} (killing rate of 50% dilution) of the H_2O_2 treatment group was between 1/6 and 1/18, while for the formaldehyde treatment group it was 1/2. Caco-2 was utilized for in-vitro neutralization assay for whole-cell bacteria toxins, anti-toxin activity level in co-incubated Caco-2 cells with H_2O_2-bacteria was slightly greater than that in the formaldehyde group, but there was no statistical difference (Fig. 4F). We speculate that some toxin-associated epitopes were still preserved in the whole-cell bacteria treated with both methods of deactivation.

3.5. H_2O_2-deactivated bacterial vaccine releases nucleic acid components and activates NF-κB signaling pathways

In this research, we aimed to assess whether H_2O_2 deactivation could enhance the immune system response in comparison to the traditional deactivation formaldehyde agent. Based on the electron microscopy results, we found that H_2O_2-deactivated bacterial vaccine released a number of unknown substances that showed transparent viscous adhesion on the surface and the surrounding matrix (Figs. 5A and S3). We suspected that this substance might contain nucleic acids that can be dissolved in water. Therefore, we incubated bacteria with Benzonase and the acquired solution was analyzed by nucleic acid electrophoresis. Results showed that the band brightness gradually weakened along with the increased concentration of the enzyme in a dose dependent manner (Fig. 5B). At the same time, the released material and the shedding of the bacteria were decreased after treatment (Fig. 5C). In addition, we used Quant-iT PicoGreen dsDNA Reagent (Invitrogen) to detect the level of DNA release, and found that the amount of dsDNA released by ATCC33591 (4000 ng/10^7 CFU) was higher than that released by PAO1 (1000 ng/10^7 CFU) (Fig. 5D, E). In order to simulate the effect of DNA release in vivo, we added 20, 40, 80 μg/ml CpG1826 oligonucleotide adjuvant to the H_2O_2-deactivated ATCC33591 incubated with DNase (40 μg/ml), the survival rates were 100% for H_2O_2-33591 vaccinated mice versus 33% for DNase-H_2O_2-33591 vaccinated mice after infection, the protection of vaccinated mice was also related to the dose of CpG (Fig. 5F, H). In order to explore the possible mechanism of immunity enhancement [20,37], we tested the effect of the H_2O_2-deactivated bacterial vaccine on the NF-κB pathway. It was revealed that HCHO-PAO1 stimulation did not significantly activate the NF-κB pathway; however, H_2O_2-PAO1 treatment led to NF-κB signal activation within 10 min. In the absence of the released nucleic acids, the phosphorylation of NF-κB was decreased in H_2O_2-PAO1 (Fig. 5G). H_2O_2 deactivation of bacteria induced stronger effect on the NF-κB pathway than HCHO-bacteria, and the observed immune enhancement is suspected to be associated with the release of nucleic acids.

4. Discussion

The new deactivation technology introduced a new direction in vaccine formulation, and H_2O_2 deactivation of virus has been found to be effective in promoting $CD8^+$ T cell immune responses [9,10]. In our study, we explored the potential application of this method on bacterial-based vaccines and found that H_2O_2 can effectively deactivate

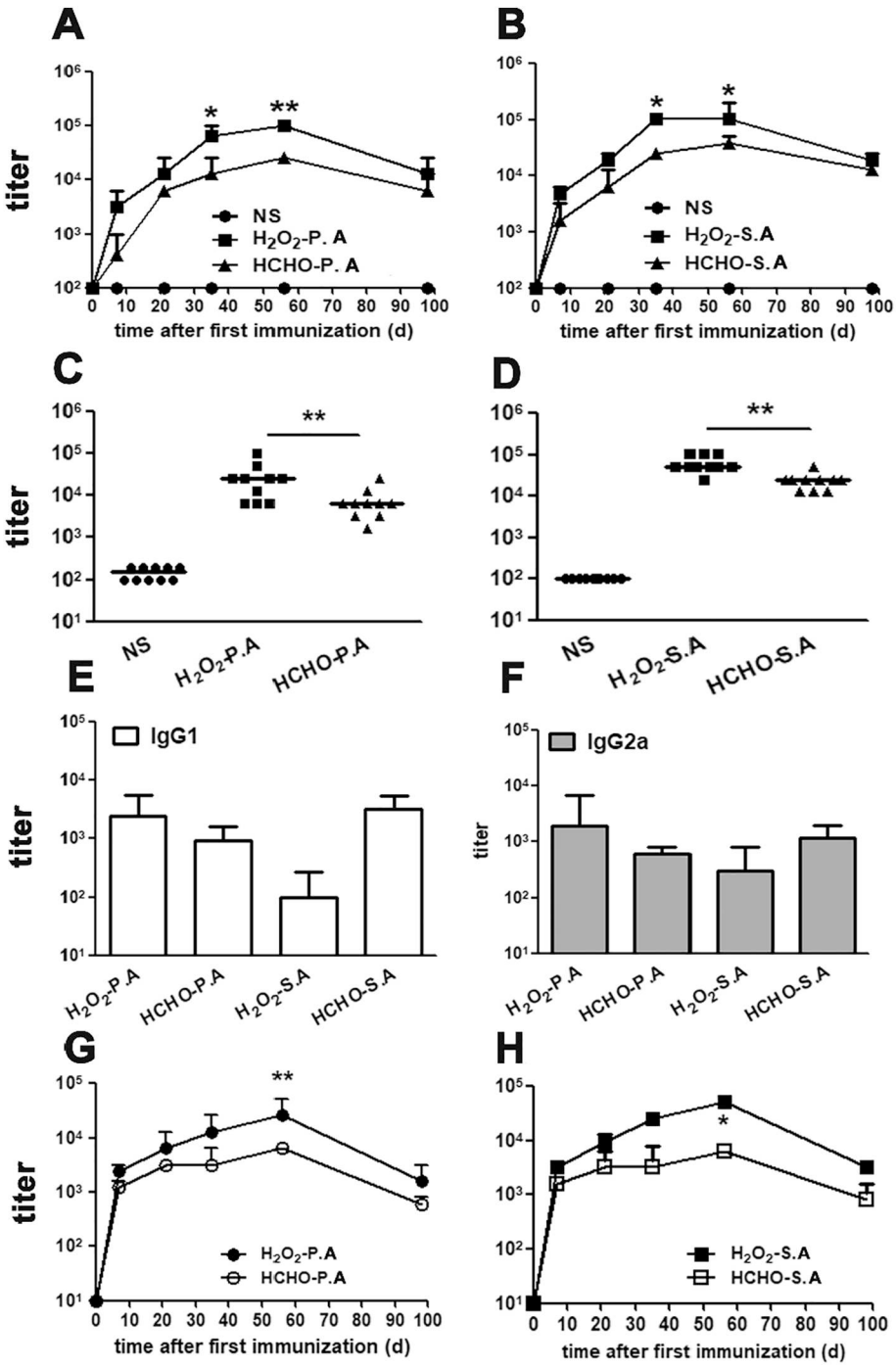

Fig. 3. H_2O_2-bacteria up-regulates the humoral immunity. Orbital venous blood was collected one week after each immunization respectively, and the anti-whole bacteria and total immunoglobulin (IgG) titer were detected in serum (A, B), the distribution of antibody titers on the 56th day after first vaccination (C, D), at the levels of 56th day after vaccination in IgG subtypes (E, F). IgG3 titers were tested for several times (G, H). Data were expressed as mean ± SEM of 10 mice analyzed each group in 3 different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the HCHO-P.A, HCHO-S.A groups. The lines in part C and D were shown the median range.

Gram-positive and Gram-negative bacteria, with a broad spectrum of antibacterial activity (Fig. 1A and B). H_2O_2 could also deactivate bacteria at low concentrations (Fig. S1). In comparison to formaldehyde deactivation, which may cause polymerization, blocking of epitopes, and induce toxicity and other side effects [29], H_2O_2 deactivation is relative safer and environmentally friendlier, as H_2O_2 is mainly decomposed into water and oxygen. Based on our results, H_2O_2-deactivated bacterial vaccines induced the expression of more antigen epitopes (Fig. 1C and D) and therefore, shorter deactivation time was required. This is of great importance as prolonged treatment time may result in reduction or even loss of immunogenicity [30]. In the current study we have established a preventive immune model [31–33] at which H_2O_2-deactivated bacterial vaccine can provide more efficient anti-infection effects at early stage (Fig. 2). The anti-PAO1 or anti-33591 whole-cell IgG titer in the H_2O_2-deactivated group was increased (Fig. 3A–D). This was possibly attributed to the retaining of more complete epitopes on the surface of bacteria in the H_2O_2-deactivated group. IgM has a protective role in bacterial infection, and is also a regulator of B cell development [30,34]. Serum obtained from immunized mice with H_2O_2-bacteria contained a high titer of IgM (Fig.

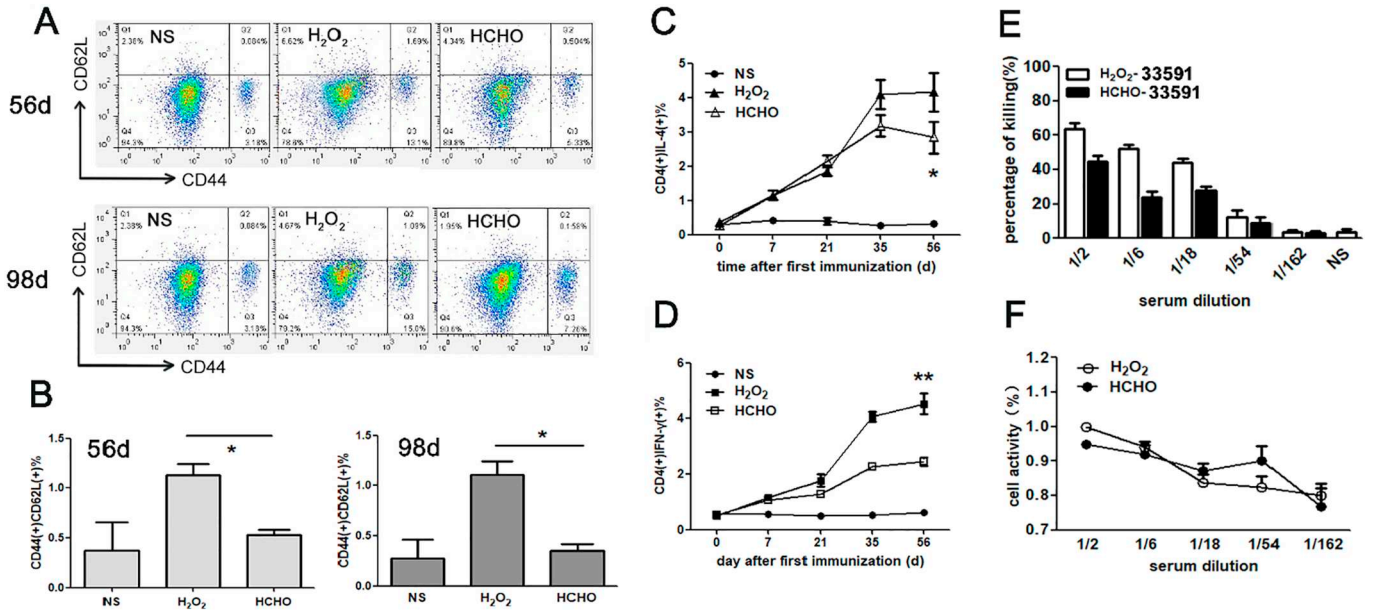

Fig. 4. H_2O_2-bacteria stimulates T cell activation and opsonic response. The memory T cell response in immunized mice (A, B). Lymphocytes were isolated from spleen 56 days after first immunization and stained with CD44, CD62L for memory T cells. H_2O_2 treatment of ATCC33591 induces activated T-cell responses (C, D). Lymphocytes were isolated from spleens and stained with CD4, IFN-γ, IL-4. Data expressed the double positive percentages and were representative of 3 mice in three independent experiments, $^*P < 0.05$, $^{**}P < 0.01$ (H_2O_2 vs. HCHO). We prepared spleen cell suspension incubated with complement, neutrophils, and viable bacteria for 90 min and cultured on LB plates for CFU counting. (E) The average value of duplicate colonies was recorded corresponding to a 50% effective concentration (EC50). (F) Cell viability was analyzed by CCK-8 assay. In brief, 5×10^4 Caco-2 cells/well were treated with PAO1 in the presence or absence of amounts of rabbit anti-PAO1 serum, then incubated at 37 °C for 4 h. Absorbance was measured at 570 nm (H_2O_2-33591 vs HCHO-33591). 10 mice were used in the opsonin phagocytosis and anti-bacterial toxins assays.

S2). H_2O_2-deactivated bacterial vaccine mainly induced IgG3, thus it could be concluded that is comparable to the IgG3-specific responses reported in *P. falciparum* malaria, which were strongly associated with immunity [35]. In our research, the IgG3 titer in H_2O_2 group was about 10 times greater than that of the formaldehyde group (Fig. 3G and H). Therefore H_2O_2 deactivation may be a new approach for immune regulation.

Enhancement of cellular immunity was observed by administration of H_2O_2 whole-cell vaccines. In *P. aeruginosa* infection, cellular immunity plays a central role in shaping the type of inflammation, with involves secretion of IFN-γ [18,31]. In this research, we found increased numbers of $CD4^+$T cells and secretion of IFN-γ, in the H_2O_2-deactived group. Collectively, these results demonstrate the advantage of H_2O_2-deaction over formaldehyde deactivation (Fig. 4C and D). However, the pathogen presentation process has not been investigated in our current study. The spleen released mainly cytokines of Th1 and Th2, indicating that H_2O_2-deactived bacteria may play an increasing influence in determining the antibody response prior to balance T cell response. Although the isotope of serum antibodies can be used as an indicator of Th lymphocyte dominance, it is difficult to say whether the H_2O_2-deactived bacteria would eventually regulate the IgG2a or IgG1 responses, further longer term studies would be required to be performed. More memory T cells were seen in the H_2O_2-PAO1 immunized mice compared with the HCHO-PAO1 immunized mice (Fig. 4A and B), we need to determine further whether these numbers are sufficient to induce bacteria-specific T cell responses and regulate the multiple inflammatory cytokines and chemokines, such as Th1-, Th2-, or Th17-associated cytokines.

Neutralizing antibodies are effective in blocking pathogen entry into host cells or direct killing the pathogen [36]. Opsonin-like antibody test confirmed that antibody-mediated killing process induced by H_2O_2-deactivated bacterial vaccine was significantly stronger (Fig. 4E). This result also shows that the neutralization of H_2O_2 group does not rely on the antibody, which could be coordinated through mobilization and recruitment of neutrophils [16,17,37,38]. After the anti-toxin neutralizing antibody reaction, the minor difference between the H_2O_2 and the formaldehyde groups may be due to the similarity of the type of toxin induced to relate Fcγ receptors (FcγRs) following administration in mice [17,20,26].

Finally, we have observed that the cells released an unknown material, which was preliminary verified as a nucleic acid containing substance, however whether it contained genomic DNA or RNA need to be investigated. Base on the Pico-Green quantitative fluorescent, the level of released DNA was detected (Fig. 5D and E). We would investigate the relationship between the immunomodulation mechanism of dsDNA and its anti-infection effect. We speculate that the released nucleic acid might be important vaccine effector molecules. In addition, bacterial DNA usually up-regulate NF-κB immune pathway, while signals from adjuvant CpG oligonucleotides [39,40] also activate the phosphorylation of NF-κB. Therefore, we hypothesized that the released nucleic acids could serve as adjuvant analogues [41] to enhance a similar protective response (Fig. 5F). The activation of the NF-κB pathway in the H_2O_2-PAO1 stimulated RAW264.7 cells (Fig. 5G) provided a feasible theoretical explanation for the underlying mechanism of hydrogen peroxide deactivation in increasing the body's anti-infection response, thus we will characterize further the released material.

We have demonstrated that H_2O_2-deactivated bacteria can retain relatively complete antigen epitopes, stimulate IgG2a, IgG1 and IgG3 response, and enhance IFN-γ and IL-4 secretion with anti-bacterial function. The absence of toxicity and the low cost of H_2O_2 deactivation are advantageous for potential application in immune preparations. In summary, H_2O_2, an innovative deactivation agent, elicits immune responses to prevent the infection; however, further studies on the stability of the preparation are required. A previous study [41] has reported that alum caused death by released cell factors or DNA to mediate adjuvant activity. In other research it was shown that exogenous H_2O_2 could induce the release of DNA from *S. sanguinis* and *S. gordonii*, however, obvious lysis of cells has not been observed [42]. These reports together with our results suggest that the regulation mechanism might implicate released DNA. In clinical application, H_2O_2

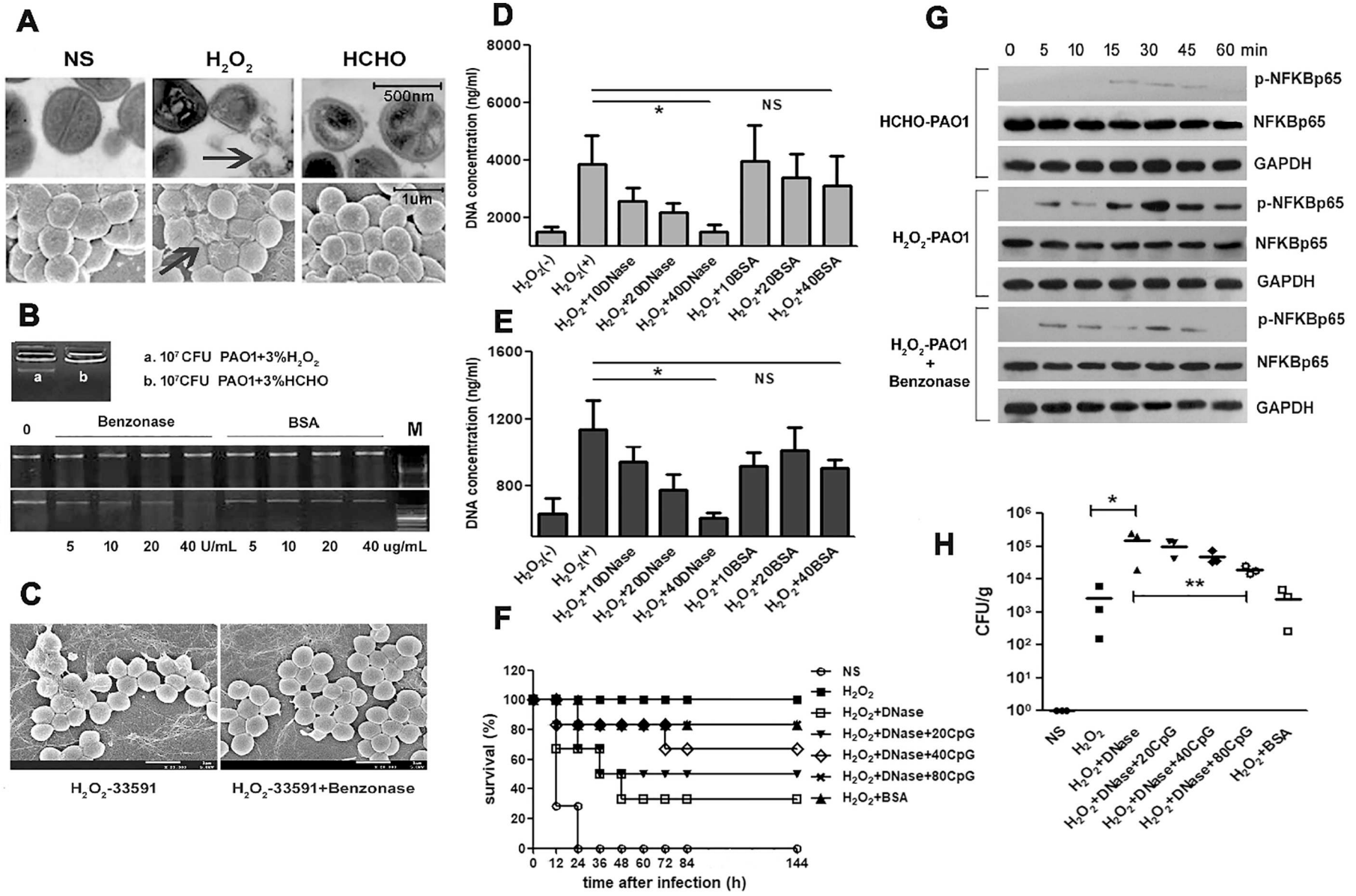

Fig. 5. Released nucleic acids of H_2O_2 treated bacteria enhance NF-κB signal.

Scanning electron microscope (SEM) was used to observe the morphological changes.

(A) Before or after H_2O_2 or formaldehyde treatment (the black arrow is shown as the material released by H_2O_2). (B) After H_2O_2 treatment, the light stripe was genomic DNA fragment from PAO1 by 0.5% agarose gel which incubated with 5, 10, 20, 40 U/ml of Benzonase (Merck), the brightness of bands was decreased while bovine serum albumin (BSA) was considered as the control of protein. Electrophoresis chart displays (B-c) before and (B-d) after nuclease treatment. (C) SEM was shown after 40 U/ml Benzonase treatment. Released nucleic acids of H_2O_2 treated bacteria was detected with Pico-Green quantitation. Genomic DNA fragment extracted by ATCC33591 (D) or PAO1 (E) incubated with 10, 20, 40 μg/ml of DNase (Invitrogen). Survival mice were demonstrated of different doses in CpG adjuvant incubation with DNase and H_2O_2 treated bacteria (F). (H) showed the splenic bacterial burden of mice. Data were mean ± SEM of three independent experiments, 10 mice were used in the survival rate assay (n = 10), $^*P < 0.05$, $^{**}P < 0.01$. (G) The HCHO-PAO1, H_2O_2-PAO1, H_2O_2-PAO1 with Benzonase (20 U/ml) stimulated RAW264.7 cells in the ratio of 20/1 whereas cell density was 5×10^6cells/well. Respectively cell lysate was collected in interval time and detected in Westernblot. Data were representative of three independent experiments.

treated whole-cell bacteria might be potentially used to establish a therapeutic vaccination to alleviate complications of drug-resistant infection.

Disclosure

The authors have no disclosures to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.055.

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