Article

PIKA as an Adjuvant Enhances Specific Humoral and Cellular Immune Responses Following the Vaccination of Mice with HBsAg plus PIKA

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An adjuvant is usually used to enhance the immune response induced by vaccines. The choice of adjuvant or immune enhancer determines the effectiveness of the immune response. Currently, aluminium (Alum, a generic term for salts of aluminium) is the only FDA-approved adjuvant. Alum predominantly induces the differentiation of Th2 cells and thus mediates an antibody immune response. Therefore, there is an urgent need for new adjuvants that enhance not only humoral but also cellular immune responses. In the present study, we demonstrates that PIKA (a stabilized dsRNA) as an adjuvant directly induces the activation and the proliferation of both B and NK cells *in vitro*. Injection of PIKA into mice results in the production of cytokines *in vivo*. In addition, the study demonstrates that PIKA promotes the maturation of bone marrow-derived dendritic cells (BMDCs) including up-regulation of the co-stimulatory molecules CD80, CD86 and CD40, and the induction of cytokines such as IL-12p70, IL-12p40 and IL-6. Importantly, after immunization of mice with HBsAg plus PIKA, the presence of PIKA enhances the titers of HBsAg-specific IgG and HBsAg-specific IFN-γ production. These results demonstrate that PIKA as an adjuvant can promote both humoral and cellular immune responses. These might have an implication in applying PIKA as an adjuvant to be used in the design and development of both therapeutic and preventive vaccines, and used in the clinical study. *Cellular & Molecular Immunology*. 2007;4(2):113-120.

Key Words: PIKA, adjuvant, Toll like receptor, HBsAg, vaccine, dendritic cell

Introduction

An adjuvant is usually used to enhance the immunogenicity of a vaccine by promoting the uptake of the immunogen and activating dendritic cells (DCs) to initiate an immune response. The classical adjuvant for vaccines, and until recently the only FDA-approved adjuvant, is Alum (1, 2). Alum provides a particle upon which the vaccine is precipitated. Although precipitation onto Alum promotes uptake of the immunogen, Alum is a poor activator of DCs and does not induce the production of IL-12 (1, 2). As a result, vaccines containing Alum initiate a Th2-type antibody response. Complete Freund's adjuvant (CFA) is another powerful adjuvant consisting of inactivated Bacillus Calmette-Guerin (BCG) and a mixture of different TLR ligands in a mineral oil solution. Although BCG is a strong activator of DCs and induces a Th1 response, CFA is not approved for human use.

Most of the vaccines currently used against infectious diseases focus on inducing the production of antibodies, which is not sufficient in all cases to protect hosts from infection of intracellular pathogens, i.e., hepatitis B, malaria, human immunodeficiency virus (HIV) and mycobacterium tuberculosis (MTB) (3). For this reason, an adjuvant will be required to promote not only humoral but also cellular

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immune responses.

Recent investigations have demonstrated that Toll like receptors (TLRs) are the critical links between the innate and the adaptive immunity (4, 5). This link, which is normally activated as a result of collaboration between adjuvants and TLRs in triggering the adaptive immunity, has been a subject of several studies. Recognition of specific pathogen associated molecular patterns (PAMPs) is mediated primarily by members of the TLR family. Stimulation through these receptors results in quantitative and qualitative changes in antigen presentation and cellular activation, thereby linking innate and adaptive immunity. An adjuvant could alert the host immune system through a mechanism similar to that of an infection by a pathogen, which involves interaction with a TLR followed by a danger signal to the immune system (6). Secretion of cytokines and regulation of the expression of co-stimulatory molecules induced by the innate response shape the magnitude and quality of the adaptive immune response.

PIKA is a stabilized dsRNA (double-stranded RNA) that interacts with TLR3 (7, 8). Although there have been reports as to the humoral immunity induced by PIKA, no studies have been reported with respect to the cellular immunity induced by PIKA (9, 10). In this study, we evaluated the immune responses induced by PIKA both *in vitro* and *in vivo*. Our finding is that PIKA induces the activation and proliferation of both B cells and NK cells as well as secretion of cytokines. PIKA directly induces the maturation of BMDCs by up-regulation of the co-stimulatory molecules *in vitro* and production of cytokines *in vivo*. In addition, as an adjuvant, PIKA enhances the HBsAg-specific humoral and cellular immune responses.

Materials and Methods

Mice

Female C57BL/6 mice and Balb/c mice were purchased from Animal Center of Sun Yet-sen University (Guangzhou, China) or Guangdong Medical Experimental Animal Center (Guangzhou, China). All animals were housed in animal care facility under pathogen-free conditions and were used at 6-8 weeks of age. All experiments were approved by our department's animal care and use committee.

Reagents

FITC labeled anti-mouse CD19 mAb (FITC-anti-CD19), APC-anti-CD25, PE-anti-CD3, FITC-anti-Ly49, FITC-anti-Ia, PE-anti-CD11c, PE-anti-CD80, FITC-anti-CD86, FITC-anti-CD40, and ELISA sets for TNF-α, IFN-γ, IL-12p40, IL-12p70, IL-6 were purchased from BD/PharMingen (San Diego, CA, USA). The adjuvant PIKA (Batch number: 200412012F) and HBsAg peptide specific for $CD8^+$ T cells were provided by NewBiomed PIKA (Guangzhou) Ltd (Guangzhou, China). HBsAg was provided by Dalian Han Xin Bio-pharmaceutical Corporation Ltd. RmGM-CSF and rmIL-4 were purchased from Peprotech (Rocky hill, NJ, USA). RPMI 1640 medium, penicillin-streptomycin and 2-mercaptoethanol were obtained from GIBCO (Grand Island, NY, USA).

Immunization protocol

Balb/c mice were immunized *i.m*. at the tibialis anterior muscles on day 0 and day 14. HBsAg (3 μg/mouse) absorbed to Alum (75 μg/mouse) or PIKA (60 μg/mouse) was directly mixed and given in a total volume of 50 μl. Animals were sacrificed after 2 weeks following the final immunization.

Cell preparation and culture

Balb/c mice were euthanized and sacrificed. After using 5 ml syringes of RPMI 1640 to rinse out the spleen cells to the peri disk, till the spleen became empty and semi-transparent and lysing RBC with hypotonic ammonium chloride solution, single-cell suspensions were prepared and resuspended in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. The cells were cultured at a concentration of 2×10^6 cells/ml for three days at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of bone marrow-derived dentritic cells (BMDCs)

BMDCs from C57BL/6 mice were prepared as described previously (11). After removing all muscle tissues from the femora of C57BL/6 mice, the bones were placed in a 60-mm dish with 75% alcohol for 1 min, washed twice with Hanks' solution, and transferred into a fresh dish. Both ends of the bones were cut with scissors, and the marrow was flushed out using 2 ml syringe and 25 gauge needle until the bones became white. The bone marrow cell suspensions were centrifuged (700 × g, 8 min at 4°C), and red cells were lysed with ammonium chloride. The cells were washed twice with Hanks' solution, and cell concentration was adjusted to 2×10^6 cells/ml and cultured with complete RPMI 1640 medium in the presence of rmGM-CSF (20 ng/ml) and rmIL-4 (5 ng/ml) in a 25 cm^2 culture flask. After 72 h, the flask was gentlely shaked and all the supernatants were replaced with complete RPMI 1640 medium containing rmGM-CSF (20 ng/ml) and rmIL-4 (5 ng/ml), and cultured for another 72 h.

Cell surface staining and flow cytometric assay

At the end of culture, cells were harvested and washed twice with PBS, and Fc receptors were blocked with anti-CD16/CD32 monoclonal antibodies at 4°C for 20 min and stained with fluorescence labeled antibodies. All incubations were conducted on ice, and washed twice with cold staining buffer after each step (PBS containing 0.1% BSA and 0.05% NaN_3). Cells were collected on a FACSCalibur (BD Bioscience, Mountain View, CA, USA), and the data were analyzed using CellQuest software (BD Bioscience, Mountain View, CA, USA).

Measurement of HBsAg-specific IFN-γ-producing cells by ELISPOT

Single-cell suspensions were prepared from spleens of mice after vaccination, and plated in 96-well microplates

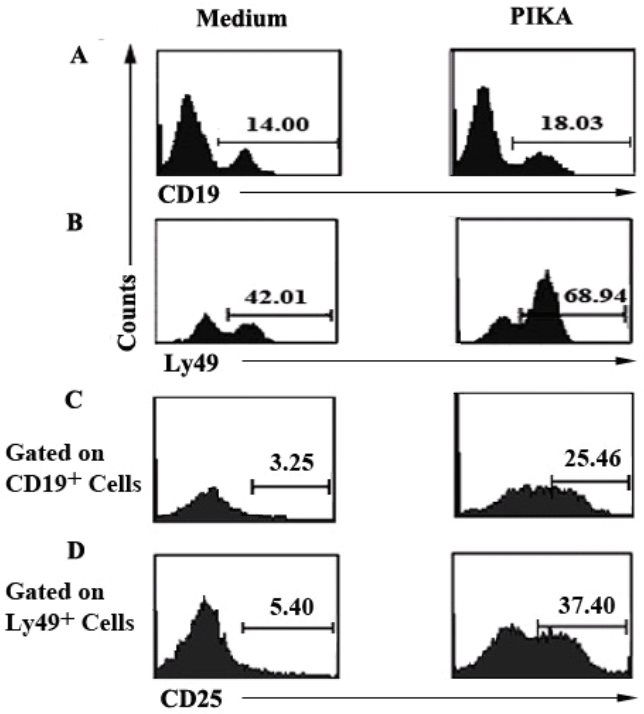

Figure 1. PIKA induced the proliferation and activation of B and NK cells in spleen. Splenocytes from Balb/c mice were incubated in the presence or absence of PIKA (250 μg/ml) for 3 days. Cells were harvested, washed and stained with surface antibodies. Lymphocytes were first gated, and the percentages of B cells (A) and NK cells (B) as well as the expression of CD25 on B cells (C) and NK cells (D) were assayed by flow cytometry. Representative results from three independent experiments with similar results were shown.

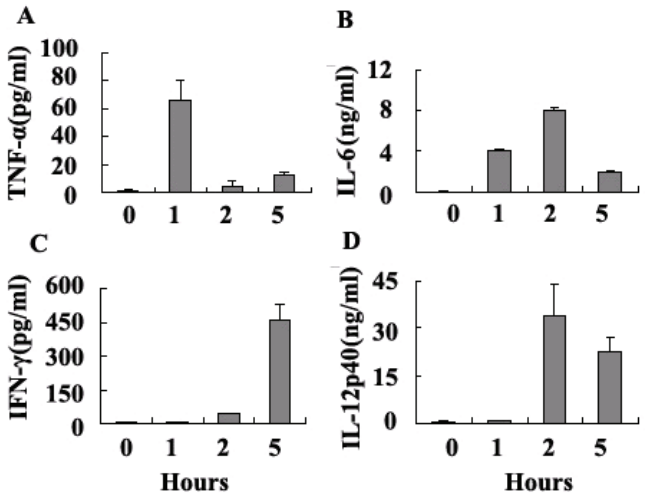

Figure 2. Kinetics analysis of cytokine production following injection of PIKA. Balb/c mice were administrated intraperitoneally (*i.p.*) with PIKA (500 μg/mouse), and sera were collected at different time points as indicated. TNF-α (A), IL-6 (B), IFN-γ (C) and IL-12p40 (D) in the sera were assayed by specific ELISAs. The data were expressed as mean ± SD (3 mice) for each time of collection point.

pre-coated with anti-IFN-γ antibody specific for ELISPOT kit (Diaclone, France) in triplicate. Cells were incubated overnight in the presence or absence of HBsAg (2 μg/ml) and anti-CD28 (1 μg/ml), or HBsAg peptide specific for $CD8^+$ T cells (10 μg/ml). The plates were then washed and alkaline phosphatase-conjugated anti-mouse IFN-γ antibody was added, developed with ready-to-use BCIP/NBT, and finally read by ChampSpot II ELISPOT reader (Sage Creation, China).

Detection of cytokines or HBsAg-specific IgG by ELISA

For the detection of cytokines in the sera or culture supernatants, IL-6 OptEIA ELISA (detection range, 31.1-2,000 pg/ml), IL-12p40 OptEIA ELISA (detection range, 15.6-1,000 pg/ml), IL-12p70 OptEIA ELISA (detection range, 62.5-4,000 pg/ml), IFN-γ OptEIA ELISA (detection range, 3.1-200 pg/ml) and TNF-α OptEIA ELISA (detection range, 15.6-1,000 pg/ml) (all from BD PharMingen) were used according to the manufacturer's protocols .

For the detection of HBsAg-specific IgG, plates were coated overnight at 4°C with 100 μl of HBsAg (2 μg/ml), and blocked with PBS containing 10% FCS for 1 h at 37°C. Diluted sera in PBS containing 10% FCS were added into wells and incubated for 2 h at 37°C. After washing, the plates were incubated with horseradish peroxidase-conjugated anti-mouse IgG (Southernbiotech, USA) at 1/8,000 dilution for 1 h at 37°C. After washing, the plates were developed with tetramethylbenzidine (TMB) and hydrogen peroxide (BD PharMingen), and read using Elx800 universal microplate reader (BIO-TEK, USA).

Statistical analysis

A non-parametric two-tailed *t* test was carried out to analyze statistical significance in the experiment groups. Statistically significant difference was considered with p value < 0.05.

Results

PIKA induced the activation and proliferation of B and NK cells

Our previous study showed that PIKA could induce IFN-γ production by murine splenocytes in a dose dependent manner *in vitro* (12). To analyze the cells that PIKA affected on, splenocytes from Balb/c mice were incubated in the presence or absence of PIKA (250 μg/ml) for 3 days. The cells were then harvested, washed and stained with surface antibodies to determine the activation and percentages of cell populations in splenocytes. As shown in Figure 1A, PIKA slightly increased the percentage of $CD19^+$ B cells (18.03%) compared with un-stimulated splenocytes (14.0%). Interestingly, PIKA markedly enhanced the proliferation of NK cells (68.94%) compared with un-stimulated splenocytes (42.01%) (Figure 1B). Furthermore, PIKA markedly induced the

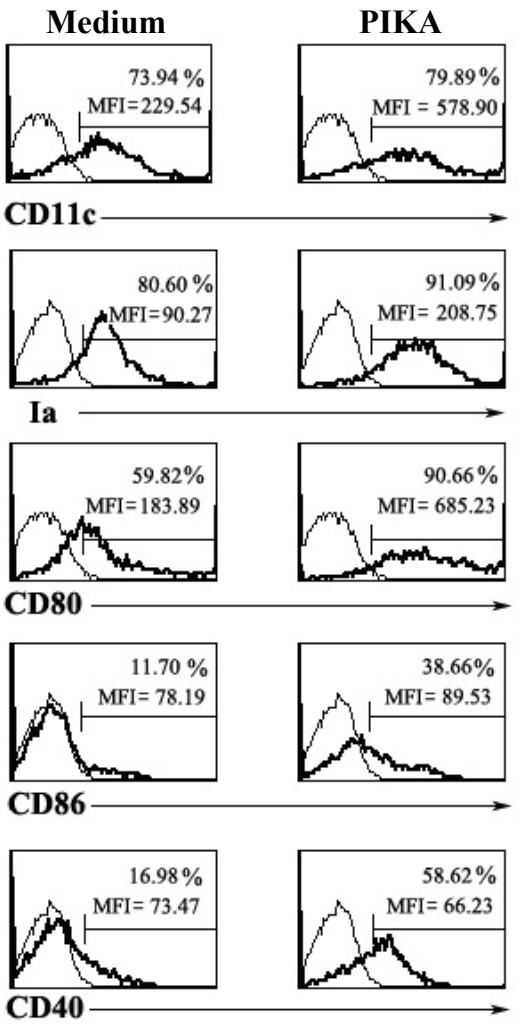

Figure 3. PIKA induced the maturation of BMDCs. BMDCs were prepared as described in Materials and Methods and cultured with or without PIKA (250 μg/ml) for 18 hours. The cells were stained with fluorescence labeled anti-CD11c, anti-Ia, anti-CD80, anti-CD86 and anti-CD40 antibodies, and determined by FACS. The percentage of positive cells and the MFI (mean fluorescence intensity) were analyzed by CellQuest software. Thin lines are the isotype control. Representative results from three independent experiments with similar results were shown.

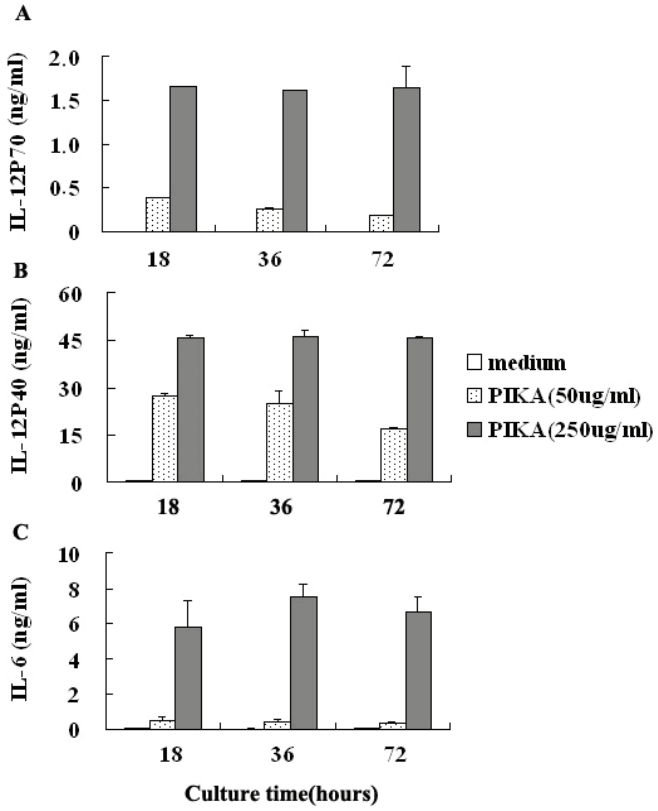

Figure 4. PIKA induced the production of cytokines by BMDCs. BMDCs were prepared as described in Materials and Methods. BMDCs were harvested, washed with medium, and cultured in the presence or absence of PIKA (at a concentration of 50 μg/ml and 250 μg/ml respectively). Culture supernatants were collected at different time points (18 h, 36 h, and 72 h). The levels of IL-12p70 (A), IL-12p40 (B) and IL-6 (C) in the culture supernatants were determined by specific ELISAs. Representative results from three independent experiments with similar results were shown.

expression of CD25 on $CD19^+$ B cells (25.46%) compared with un-stimulated cells (3.25%) and on NK ($Ly49^+$) cells (37.40%) compared with un-stimulated splenocytes (5.40%) (Figures 1C and 1D). However, PIKA had no significant effect on the activation of $CD4^+$ and $CD8^+$ T cells (data not shown).

Kinetics analysis of cytokine production following PIKA injection

We next determined the production of cytokines following PIKA injection *in vivo*. Balb/c mice were injected *i.p.* with PIKA (500 μg/mouse), and sera were collected at different time points following administration as indicated in Figure 2. The levels of cytokines in the sera were determined by ELISA. As shown in Figure 2, injection of PIKA resulted in the production of TNF-α, IL-6, IFN-γ and IL-12p40. However, the amount and timing of production of each cytokine varied. Following the administration of PIKA, TNF-α reached the peak level at 1 h (Figures 2B and 2D), IL-6 and IL-12p40 at 2 h and IFN-γ at 5 h (Figure 2C).

PIKA induced the maturation of BMDCs

To determine whether PIKA could induce the maturation of DCs, immature BMDCs were prepared as described in Materials and Methods, and were harvested at day 6. After washing, immature BMDCs were cultured with PIKA for 18 h. The cells were collected, washed and incubated with fluorescence labeled anti-CD11c, anti-Ia (MHC II), anti-CD80, anti-CD86 and anti-CD40 antibodies. The expression of the molecules on BMDCs was determined by FACS. As shown in Figure 3, PIKA had no significant effect on the

percentage of $CD11c^+$ and Ia^+ cells compared with un-stimulated BMDCs. However, PIKA markedly increased the MFI (mean fluorescence intensity) of CD11c and Ia (MHC II) expression (Figure 3). In regarding to the expression of co-stimulatory molecules, PIKA markedly increased the percentages of $CD80^+$ and $CD40^+$ cells and the MFI of CD80, CD86 and CD40 on BMDCs (Figure 3), indicating that PIKA promoted the maturation of BMDCs.

PIKA induced the production of cytokines by BMDCs

The cytokines produced by DCs are likely to influence the differentiation of Th1 and Th2 cells. Therefore, we analyzed whether PIKA could activate DCs to produce cytokines following PIKA stimulation. BMDCs were stimulated with or without PIKA at different concentrations (50 μg/ml and 250 μg/ml, respectively). As shown in Figure 4, PIKA significantly induced the production of IL-12p70 (Figure 4A), IL-12p40 (Figure 4B) and IL-6 (Figure 4C) by BMDCs in a dose-dependent manner. After incubation with PIKA for 18 h, these three cytokines reached the highest levels. There was undetectable amount of IL-12p40 and IL-12p70 production in un-stimulated BMDCs. The similar results were observed in IL-6 production by BMDCs (Figure 4).

PIKA increased HBsAg-specific IgG production following prime and boost administration with HBsAg plus PIKA

Balb/c mice were immunized with HBsAg in combination with PIKA to further study whether PIKA could enhance adaptive immunity during administration with antigen. As a control, mice were immunized with HBsAg plus Alum as described in Materials and Methods. As shown in Figure 5A, immunization of mice with HBsAg plus PIKA markedly increased HBsAg-specific IgG production compared with mice immunized with HBsAg alone or with HBsAg plus Alum ($p < 0.05$). It was further observed that PIKA increased both HBsAg-specific IgG2a (greater than > 20 times increase of difference in titers with PIKA) and IgG1 (approximately 3 times increase of difference in titers with PIKA) production with predominantly increase compared with HBsAg plus Alum (Figures 5B and 5C).

HBsAg-specific IFN-γ production was elicited following prime and boost administration with HBsAg plus PIKA

To assess the cellular immune response induced by PIKA, splenocytes from immunized mice were cultured at a density of 3×10^5 cells/well with HBsAg (2 μg/ml) and anti-CD28 (1 μg/ml) (Figure 6A), or HBsAg peptide specific for $CD8^+$ T cells (10 μg/ml) (Figure 6B) in the 96-well pre-coated ELISPOT plate. As shown in Figure 6, the frequency of HBsAg-specific IFN-γ producing cells was increased following HBsAg plus PIKA administration compared with mice immunized with HBsAg alone or HBsAg plus Alum ($p < 0.05$).

Discussion

An adjuvant is usually used to enhance the immunogenicity

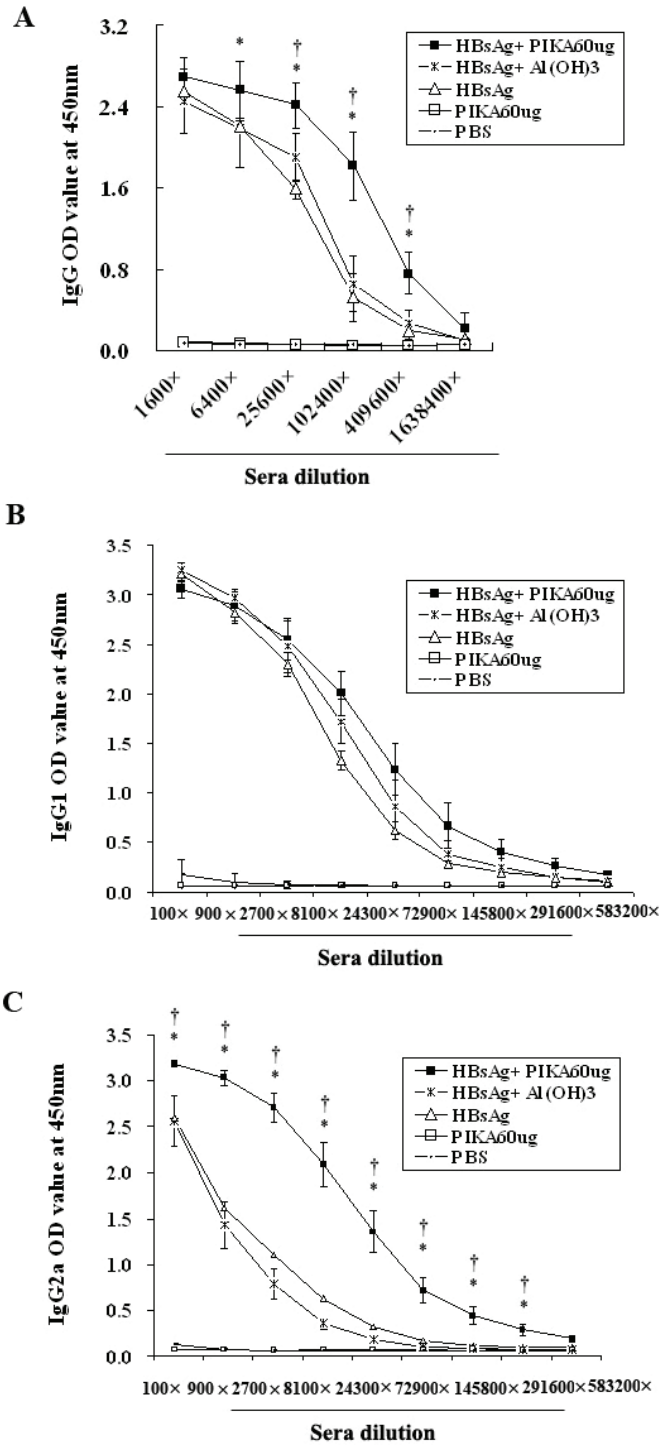

Figure 5. PIKA increased HBsAg-specific IgG production following prime and boost with HBsAg plus PIKA administration. Mice were vaccinated as described in Materials and Methods. Sera from immunized and non-immunized mice were collected two weeks after the final immunization. The titers of IgG (A), IgG1 (B) and IgG2a (C) antibodies specific for HBsAg in sera were determined at various dilutions by ELISA. Data were expressed as mean ± SD (6 mice) for each group. A non-parametric two-tailed *t*-test was used for statistical analysis. *, $P < 0.05$, compared with HBsAg immunized group. †, $p < 0.05$, compared with HBsAg plus Alum immunized group.

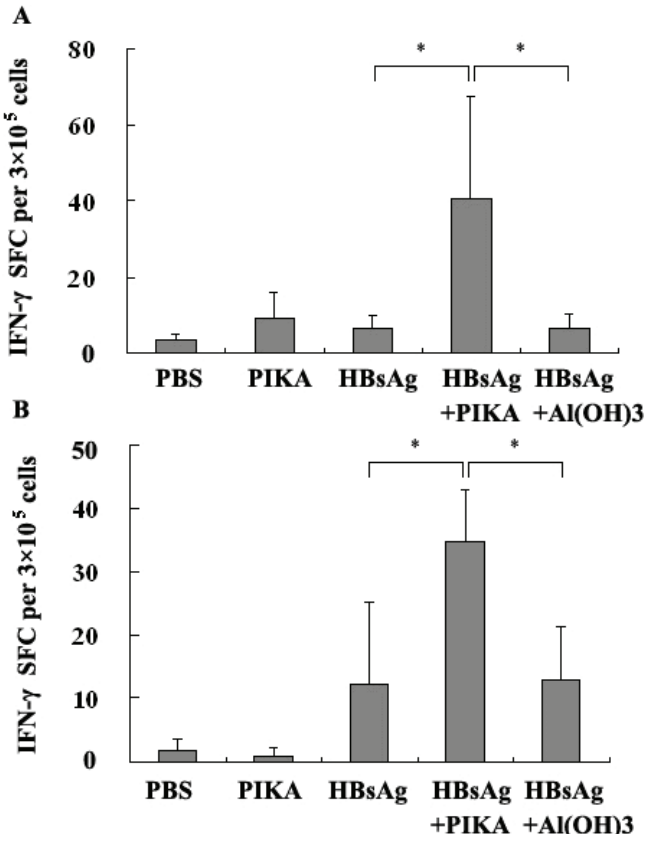

Figure 6. HBsAg-specific IFN-γ production is elicited following prime and boost with HBsAg plus PIKA administration. Mice were vaccinated as described above. Two weeks after boost, splenocytes were prepared and cultured at a density of 3×10^5 cells/well with HBsAg (2 μg/ml) and anti-CD28 (1 μg/ml) (A), or HBsAg peptide specific for $CD8^+$ T cells (10 μg/ml) (B) in the 96-well pre-coated ELISPOT plate. After incubation for 16 h, ELISPOT assay was performed as described in Materials and Methods. Data were expressed as mean ± SD for each group (6 mice). *, $p < 0.05$, compared with HBsAg alone or HBsAg plus Alum immunized group.

of a vaccine by promoting the uptake of the immunogen and activating DCs to initiate the immune response. Regardless of the vaccine target, successful immunization results in the activation of the adaptive immunity, which might be accomplished, in part, through the stimulation of the Toll-like receptors (TLRs) (5, 13). TLRs are a family of pattern-recognition receptors that recognize structural components shared by many bacteria, viruses and fungi. PIKA is a stabilized dsRNA that was originally developed as a post-infection vaccine against rabies (9, 10). dsRNA is a universal viral molecular pattern and activates an immune response through recognition by TLR3. Another important dsRNA-signaling pathway is initiated by the RNA helicase RIG-I (7, 8, 13).

NK cells are major players in the antiviral immune response. NK cells express TLR3 and are activated directly in response to poly I:C (15-17). In our present study, we showed that PIKA could markedly induce the expression of CD25 and promote the proliferation of NK cells *in vitro*. Previous studies showed that treatment of highly purified NK cells with poly I:C significantly augmented NK cell-mediated cytotoxicity. Poly I:C stimulation also lead to the up-regulation of the activation marker CD69 on NK cells. Furthermore, NK cells responded to poly I:C by producing pro-inflammatory cytokines such as IL-6 and IL-8, as well as the antiviral cytokine IFN-γ (15). Similarly, our previous study showed that PIKA could induce IFN-γ production by murine splenocytes in a dose dependent manner *in vitro* (12).

Dendritic cells (DCs) link between the innate and the acquired immune system by presenting antigens and by their expression of pattern recognition receptors that detect microbial molecules in their local environment. Whether the adaptive immune system ignores presented antigens or responds to antigens through tolerance or the induction of effector cells most likely depends on the maturation state of antigen-presenting DCs (5). Therefore, we determined whether BMDCs could be activated by PIKA. Our results showed that PIKA could induce the maturation of BMDCs assessed by up-regulation of co-stimulatory molecules CD80, CD86 and CD40, and significantly induced the production of cytokines including IL-12p40, IL-12p70 and IL-6 in a dose-dependent manner by BMDCs. In line with Janeway's insightful hypothesis on "signal 2" regulation by innate recognition, co-stimulatory molecules are rapidly up-regulated on DCs in response to TLR ligands, converting "immature DC" into "mature" cells that possess an increased ability to stimulate T cell proliferation (6). Another study showed that poly I:C, a ligand for TLR3, also induced IL-12p70 production by some types of human DCs (19). IL-12 is a heterodimeric pro-inflammatory cytokine that regulates T-cell and NK-cell responses, induces the production of interferon-γ (IFN-γ), favors the differentiation of T helper 1 (Th1) cells and is an important link between innate resistance and adaptive immunity (18). Inducing an antigen specific Th1 and $CD8^+$ T cells immune response protects hosts from infection of intracellular pathogens, i.e., hepatitis B, malaria, human immunodeficiency virus (HIV) and mycobacterium tuberculosis (MTB) (3).

Production of pro-inflammatory cytokines such as TNF-α, IL-6, IFN-γ and IL-12p40 was observed following the injection of PIKA. In line with the reports of Ulrike Wille-Reece, et al. on R848 and CpG (20), we demonstrated that as early as 1 h following the administration of PIKA, the presence of pro-inflammatory TNF-α reached a peak level immediately, however, IL-6 reached peak levels at 2 h. The amount of IFN-γ and IL-12p40 produced following the injection of PIKA *i.p.* was crucial for inducing a Th1 and $CD8^+$ T cell responses.

Hepatitis B virus infection remains one of the important worldwide health problems, particularly in China where there is an estimated 130 million carriers of the virus and approximately 30 million chronic patients (21, 22). A preventive or therapeutic vaccine with HBsAg as the antigen (or DNA vaccine expressing HBsAg) aimed at inducing or

increasing HBV-specific CD8 and CD4 T cell response has been tested in animal models and in human clinical trials (23-25). Following prime and boost vaccination with HBsAg plus PIKA, HBsAg-specific IgG production was significantly increased compared with mice immunized with HBsAg alone or with HBsAg plus Alum ($p < 0.05$). Further studies on the immunoglobulin isotype demonstrated that both HBsAg-specific IgG2a and IgG1 production were enhanced (Figures 5B and 5C). Analysis of the cellular immune response indicated that HBsAg-specific IFN-γ production was enhanced in mice vaccinated with HBsAg plus PIKA compared with mice immunized with HBsAg alone or HBsAg plus Alum ($p < 0.05$). These results indicated that PIKA could markedly induce HBsAg specific cellular immune response. Consistent with our study, Lin HX, et al. reported that PIKA (termed PICKACa by the author) protected mice against peripheral infection with both fixed and wild rabies strains. PIKA also enhanced the protective activity of an experimental rabies vaccine injected either before or after rabies infection. PIKA enhanced both the non-specific immune responses and specific immunity including antibody production and cell mediated immunity as assessed by IL-2 production (10).

Taken together, the study's findings demonstrate that the PIKA is a new adjuvant which induces HBsAg-specific humoral and cellular immune responses following prime and boost administration with HBsAg plus PIKA. This effect may be due to enhancing antigen presentation by APCs by up-regulation of co-stimulatory molecules on DCs, and inducing production of pro-inflammatory cytokines by affecting on innate cells such as NK cells and DCs. The research data imply that PIKA is suitable as an adjuvant for use in the design and development of both therapeutic as well as preventive vaccines and may be applicable for use in clinical studies.

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