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Augmented humoral and cellular immune response of hepatitis B virus DNA vaccine by micro-needle vaccination using Flt3L as an adjuvant

Qi Zhou, Fang Wang, Fu Yang, Yue Wang, Xiaoying Zhang, Shuhan Sun*

Department of Medical Genetics, Second Military Medical University, 800 Xiang-Yin Road, Shanghai 200433, PR China

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ABSTRACT

The purpose of this work was to assess the immune response against plasmid DNA encoding hepatitis B virus (HBV) HBsAg in C57BL/6 mice inoculated by micro-needle. Fms-like tyrosine kinase 3 ligand (Flt3L), a hematopoietic growth factor, was used as an adjuvant. Immune response was determined by analysis of cytokine production. In addition, cytotoxic lymphocyte (CTL) activity was measured 7 days after *in vitro* addition of tumor cells (CT26/S) stabling expressing HBsAg. The efficacy of immunoprotection against CT26/S was determined by antibody response to this challenge. A strong antibody response was induced by inoculation with the DNA vaccine via micro-needles assisted with Flt3L, Administration of the vaccine to splenocytes induced significantly higher levels of interleukin-12 and gamma interferon. CTL response to the vaccine was stronger than that stimulated by intramuscular or micro-needle injections alone. Our study suggests useful strategies for improving the efficacy of vaccines against HBV and other pathogens.

1. Introduction

Development of a safe, effective and affordable vaccine represents the best hope for controlling the worldwide spread of hepatitis B virus (HBV). DNA vaccines offer a promising alternative to conventional vaccines. DNA vaccines been shown to elicit humoral and cellular immune responses against some viral, bacterial and parasitic pathogens [1,2].

In a previous study, we found that intramuscular injection of a plasmid DNA vaccine induced a humoral immune response that protected tree shrews against HBV infection [3]. However, clinical trials have demonstrated that the immune response stimulated by a DNA vaccine does not provide full immunity [4,5]. This mode of antigen delivery is not efficient and does not induce an optimal immune response. This limitation is primarily due to: (1) the low number of dendritic cell (DCs) and other antigen-presenting cells (APCs) that can be transfected by naked DNA and (2) the inability of these cells to engage innate immune effector mechanisms critical for the generation of adaptive immunity [6,7].

DCs play a central role in the orchestration of the primary and memory phase of immune response. Many cytokines regulate the number and differentiation of DCs *in vivo*. Fms-like tyrosine kinase 3 ligand (Flt3L) is a hematopoietic growth factor that plays a unique role in the immune response through induction of large numbers of DCs and natural killer cells (NK) [8,9]. Brasel et al. found that culturing with Flt3L for 8 days caused generation of DCs from BM progenitors. These DCs expressed high levels of co-stimulatory and major histocompatibility complex (MHC) molecules which caused a potent allogeneic T cell response. The T cells and were able to process and present protein antigens to antigen-specific $CD4^-$ T cells [10]. These data suggested that Flt3L activated DCs, resulting in an improved immune response. However, there is a low abundance of DCs in the peripheral tissue.

Skin is a highly immunoreactive tissue that contains abundant DCs and other APCs in the epidermis [11]. Conventional intramuscular injection of a DNA vaccine does not induce an immune response in the epidermis. Logic suggests that the immune response would improve if the vaccine were injected into the epidermis. The vaccine delivery problem calls for a cheap and effective intracutaneous antigen delivery technology.

Micro-needles are micro-fabricated polymer projections that mechanically disrupt the skin and delivers vaccines to the epidermis. DNA is then readily taken up by affluence APCs, leading to powerful antigen presentation in regional inductive immune sites. Micro-needles have been approved for many drug-delivery systems and have been used in various immune studies [12–14].

In this study, we compared immune responses to intramuscular or micro-needle injection of plasmid DNA encoding HBsAg. Flt3L was used as an adjuvant to induce HBsAg-specific immunity. Our studies demonstrate that immunization by micro-needles is more effective than the traditional method of vaccine delivery.

* Corresponding author. Tel.: +8621 81871053; fax: +8621 81871053.
E-mail address: shsun@vip.sina.com (S. Sun).

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2. Methods

2.1. Plasmid construction

The plasmid pVAX-S encoding HBV small envelope protein was constructed, amplified and purified as described in our previous work [3]. The plasmid pVAX-Flt3L (short to pFlt3L) was constructed by cloning the cDNA of Flt3L into the BamHI/XbaI sites of the eukaryotic vector pVAX. This recombinant pFlt3L was amplified in *Escherichia coli* TOP10 strain, isolated by alkaline lysis, purified by double banding in cesium chloride equilibrium gradients and conformed by DNA sequencing. Endotoxins were removed by multiple Triton X114 (Sigma–Aldrich, Steinheim, Germany) extractions, with a consequent plasmid preparation containing fewer than 50 endotoxin units per milligram of purified DNA, which was detected by limulus amebocyte lysate assay (A&C, Zhanjiang, China).

2.2. Micro-needles preparation and analysis

Solid micro-needles were etched from silicon substrates as described previously (Henry et al., 1999). Briefly, silicon wafers were used as the starting material. The wafers were cleaned in a solution 2 part by volume deionized water, 1 part 25% hydrogen peroxide, and 1 part 75% sulphuric acid at 100 °C for 15 min, and then clean silicon wafers by deionized water for 5 min silicon wafers through wet and dry oxygen alternating oxidation process formed silicon oxide surface layer thickness of about 2 μm. A layer of photosensitive material (1827 photoresist), was deposited onto the silicon wafers. The wafer and photoresist were then exposed to ultraviolet (UV) light through the mask by means of an optical mask aligner (Hybralign Series 500, Optical Associates, Inc., Milpitas, CA). In the subsequent step, Reactive ion etching (RIE; Plasma Therm, St. Petersburg, FL) was then carried out with 20 standard cm^3/min (sccm) SF_6 and 15 sccm O_2 at a pressure of 20 Pa and power of 150 W for a run time of about 200 min. Micro-needles fabrication were finished when the silicon formed a five-pointed star-shaped needles [15–17].

To confirm the validity of micro-needles we used, C57BL/6 mice were (n = 5) immunized Renilla luciferase plasmid by micro-needles at the left hind limb of mice and the right hind limb as control, mice were sacrificed 2 weeks post-immunization and hind limbs was removed aseptically, muscle of immune sites and control were chipped into nubble and grinded with liquid nitrogen, tissue homogenate centrifuge with maximal rotation rate 5 min at 4 °C, the supernatant were then harvested for measurement of their renilla luciferase activities with a Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (BERTHOLD, Wildbad, Germany). The experiments were performed three times, and the P-value was evaluated by Scheffe's F-test.

2.3. Stability of micro-needles and structural integrity of vaccine on micro-needles

To confirm the stability of micro-needles we used, C57BL/6 mice were (n = 5) immunized Renilla luciferase plasmid at the left hind limb of mice by one micro-needle and the right hind limb by another. The experiment methods were same to micro-needles analysis. And the DNA vaccine uploads on micro-needles was been washed form micro-needles at different times (12, 24, 36, 48, and 60 h) to check the structural integrity of DNA vaccine by agarose gel.

2.4. Plasmid Flt3L recruit DCs at the site of inoculation

We initiated studies to determine whether delivering plasmid Flt3L would lead to increased recruitment DCs at the site of vaccine inoculation. We therefore assessed the local cellular inflammatory infiltrates following intramuscular injection of plasmid DNA vaccines with or without plasmids expressing Flt3L. Groups of C57BL/6 mice (n = 5 per group) were immunized intramuscularly with PBS or 50 μg plasmid DNA vaccine expressing HBsAg. Certain DNA vaccinated groups were coimmunized with 50 μg plasmid Flt3L. Sufficient sham plasmid was included to keep the total dose of DNA per animal constant. The injected muscles were excised on day 7 following immunization.

2.5. Mice handing and sample collection

All the C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). They were bred and humanely cared under SPF condition in the Laboratory Animal Center of the Second Military Medical University. They were fed with a certified standard diet and tap water ad libitum. Temperature and humidity were regulated at 21–22 °C and 35–15%, respectively. Alight cycle of 12 h on/12 h off was established.

Fifty of female C57BL/6 mice were randomly allocated into five groups, the vaccination regimens were shown in Table 1. The route of micro-needle administration was direct application of the micro-needle extraction to shaved intact skin. This rote of administration is strand for the assessment of the dermal irritation potential. On the day before application, hair on animals was remove form the dorsal and trunk area. On the day of dosing, but prior to application, the animals were examined for health and the skin checked for abnormalities. No pre-existing skin irritation was observed. The DNA vaccines and control were administered into quadriceps femoris of mice three times at biweekly intervals. Intramuscular injection of 100 μg DNA vaccine was performed in quadriceps. Each injection was carried out under anaesthesia.

At the indicated times after immunization (4, 8, 12, 16, and 20 weeks), blood were obtained from the retro-orbital venous plexus under light diethylether anaesthesia. The blood of each group vaccinated mice were only collected at 20 weeks after the post-immunization. These were collected into standard vials and allowed to clot at room temperature for 30 min, and then the serum was separated by centrifugation and stored in −80 °C until analysis.

2.6. Specific antibody analysis

HBsAg-specific total antibody was analyzed by ELISA in serum from individual mice in each group with a commercial skit (SIIC KeHua Biotech co. Ltd.). Ninety-six-well plates were incubated with rHBsAg in PBS. The solid phase was incubated at 4 °C with HBsAg-specific antibody followed by incubation with horseradish peroxidase (HRP)-conjugated HBsAg. Serum from animals immunized with empty vector was used as the negative control. Binding of antibodies was measured as A_{450} after reaction of immune complexes with substrate for HRP. Total antibody in serum was converted to mIU by comparison with a serially diluted standard antibody (Centers for Disease Control and prevention China).

Table 1
The experiment group and immunization strategy of the immunized mice.

Group no.	DNA and adjuvant	Strategy	No. of mice
I	pVAX1	i.m.	10
II	pVAX(S)	i.m.	10
III	pVAX(S)	Micro-needles	10
IV	pVAX(S) + pVAX(FL)	i.m.	10
V	pVAX(S) + pVAX(FL)	Micro-needles	10

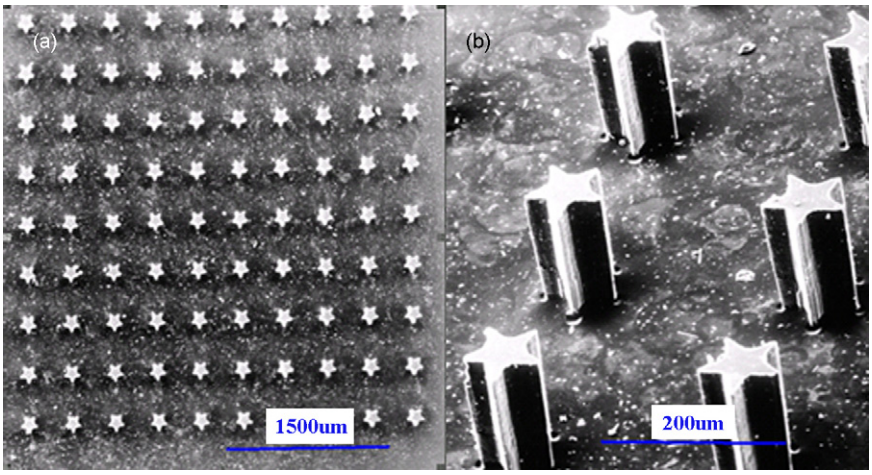

Fig. 1. Scanning electron micrographs of micro-needles made by the reactive ion etching technique: (a) a section of a 20 by 20 array of micro-needles and (b) close-up view of a micro-needle tip. Micro-needles are uniform in size and their tips, which is important for easy insertion to a desired depth in skin.

2.7. Cytokine production and assays

Vaccinated mice were sacrificed 4 weeks after the final immunization, and spleens were removed aseptically. Splenocytes from mice in each group were tested for cytokine response to purified recombined HBsAg protein (10 μg ml^{-1}). Supernatants were harvested after 24 for IL-4 and 72 h for IFN-γ, when peak amounts of the cytokine could be measured. Supernatants from at least three separate wells were pooled and assayed for the presence of IFN-γ by ELISA, using a commercial kit (Jingmei biotech). A range of dilutions of purified recombinant mouse IL-4 and IFN-γ was included as strands.

2.8. In vitro cytotoxicity assay

2.8.1. Immunomagnetic enrichment of CD8$^+$ lymphocytes

Splenocytes from mice in each group were pooled and tested for the ability to generate CTLs at week 3 after the post-immunization. The immunomagnetic depletion or enrichment of CD4$^+$ and CD8$^+$ lymphocytes was performed by a magnetic-activated cell sorter, using microbeads conjugated with rat anti-mouse CD4 (Miltenyi Biotec) and rat anti-mouse CD8 mAb (Miltenyi Biotec) according to the manufacturer's instructions (http://www.miltenyibiotec.com/). The preparation yielded enriched CD8$^+$ lymphocyte populations of approximately 95%, as confirmed by FACS analysis.

2.8.2. HBsAg-expressing CT26 cells

CT26 cells, a murine colon carcinoma cell line (H-2d, BALB/c mice), were permanently transfected with plasmid pcDNA3-(S), under continuous selective pressure with 400 μg G418 ml^{-1} (Gibco-BRL). G418-resistant and HBsAg-expressing clones designated CT26/S were selected and screened for by PCR amplification of the integrated gene and expression of HBsAg by ELISA in our previous work [3].

2.8.3. CTL response assays

Stable transfectant CT26/S cells were used to raise CTLs by *in vitro* simulation of splenocytes from immunized mice of each group for 7 days. CTL effector cells (2×10^5) were coculture with the 2×10^3 CT26 cells (transfected with the vector DNA without insert) or stable CT26/S transfectants as targets in 200 μl wells for 4 h. Specific cytolytic activity of CTLs was tested by a non-radioactive and colorimetric assay, an alternative to the ^{51}Cr-release assay for the quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase activity released from the cytosol of damaged cell into the supernatant with the Cytotoxicity detection kit (Roche). The procedure is described in the manufacturer's protocol (http://www.roche-applied-science.com/pack-insert/1644793a.pdf). The percentage of lysis was calculated as described [18].

2.9. Protection studies in vivo

Fifty C57BL/6 mice divided into five groups were used for protection study *in vivo*, the vaccination regimens were shown in Table 1. Ten C57BL/6 mice of each group were subcutaneously inoculated with 2.0×10^6 CT26/S tumor cells after the third immunization. The vitality of individual mouse was monitored for 105 days (15 weeks) after inoculation. Kaplan–Meier's curve was generated to show survival rate and survival of each group [19].

2.10. Statistical analysis

The Kruskal–Wallis non-parametric rank sum test and Wilcoxon's matched pairs test were used to test the significance of differences in humoral and cellular immune response. Analysis of variance (ANOVA) was also performed. Data were analyzed using SAS 8.2 (SAS Institution Inc.) A P-value <0.05 was considered significant.

3. Results

3.1. Characterization and efficacy of micro-needles

Micro-needles fabrication was finished when the silicon formed five-pointed star-shaped needles (Fig. 1). The left hind limb was inoculated with Renilla luciferase plasmid DNA by micro-needles or by intramuscular injection. We found that mice injected with micro-needles had a significantly higher luciferase activity than

Table 2
Renilla luciferase activity of mice hind limb (P-value <0.01).

Mice sample	Left hind limb Renilla luciferase activity	Right hind limb Renilla luciferase activity
1	13958	242
2	45580	259
3	37003	308
4	42586	341
5	46599	262

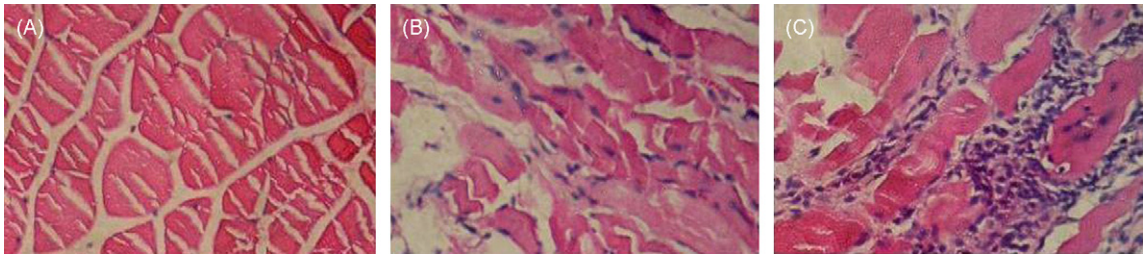

Fig. 2. Histopathology of injection sites. C57BL/6 mice ($n = 5$ per group) were immunized with PBS (A); pVAX(S) DNA vaccine alone (B); or pVAX(S) DNA vaccine with pFlt3L (C). We injected 50 μg of each plasmid with sufficient sham plasmid to keep the total DNA dose per mouse constant. Muscle sections (2 μm) were stained with H&E on day 7 following immunization. Magnification 40×.

their right hind limb (P-value <0.01), which was the control intramuscular injection of the same DNA (Table 2). These results demonstrate that micro-needles can successfully penetrate the stratum corneum and deliver drugs to the site of insertion.

3.2. Flt3L recruits DCs at the site of inoculation

Injected muscles were excised 7 days after immunization. Serial sections (2 μm) were examined for the presence of local inflammatory infiltrates (Fig. 2). Small infiltrates were observed following injection of the DNA vaccine alone. In contrast, large cellular infiltrates were recruited by the adjuvant pFlt3L. We found that administration of pFlt3L caused over a 10-fold increase in inflammatory cell recruitment compared with the DNA vaccine alone.

3.3. Stability of micro-needles and structural integrity of hepatitis B

Five mice were immunized with Renilla luciferase plasmid DNA, the left hind limb by one micro-needle and right by another. We found that there were no significant deviation between different hind limb and different mice (P-value >0.05) (Fig. 3A). And hepatitis B DNA vaccine was washed form micro-needles after upload for different times, then the DNA was check by 1% agarose gel (Fig. 3B). The pVAX-S vaccine on agarose gel was close to 3500 bp, we found after upload on micro-needles for 12–60 h there was no migration of the strap and the strap still unity no other straps emerge on agarose gel, also the gray scale of the strap no significant change between different times. These results demonstrate that micro-needles were stability and the structure and concentration of DNA vaccine upload on micro-needles was stable for a long time.

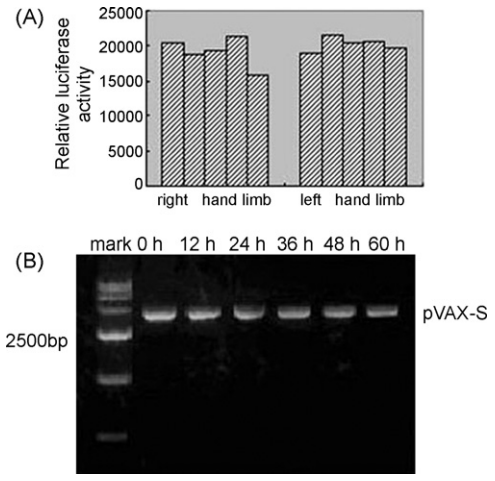

Fig. 3. The Renilla luciferase activity of C57BL/6 mice (A), the right hind limb was immunized Renilla luciferase plasmid DNA 50 μg by micro-needles and the right hind limb was delivered 100 μg. The structural integrity of hepatitis B in micro-needles was determined at different times after upload on micro-needles by gel, lane 1 was 15000 bp Mark, lane 2 was no-load DNA vaccine and lanes 3–7 were vaccine upload on micro-needles at different times (B).

3.4. Antibody titers in the blood sera of immunized animals

In order to assess immune response to different regimens (Table 1), anti-HBsAg antibody in the sera was quantitated with ELISA at several post-immunization time points (Fig. 4). Titer of the anti-HBsAg antibody in the serum began to increase on day 28 post-immunization, reaching a maximum at week 12. A significantly higher level of specific antibody was induced when pFlt3L was used as an adjuvant. The peak antibody titer for the micro-needles group was approximately 3-fold higher than the peak titer for the intramuscular group.

3.5. Cytokine production by different combinatorial vaccinations

As a measure of the cellular immune response elicited in the immunization strategies described above. We quantified the production of the cytokines IFN-γ and IL-12 released form splenocytes from five immunized mice re-stimulated with purified rHBsAg *in vitro*. Fig. 5 shows that mice immunized with DNA vaccine adjuvant with pFlt3L by micro-needles (group V) elicited a significant enhancement of IFN-γ and IL-12 production, even significantly higher than that of regiment with three round pVAX(S) alone (Table 1, group V versus group II, $P < 0.01$). Suggesting this combination resulted in stronger Th1-type cellular immune response.

3.6. Micro-needles facilitated HBsAg-specific CTL response

The antigen-specific CTL response was measured in splenocytes from five mice in each group. Mice immunized by micro-needles induced significantly higher CTL responses than that induced by i.m. (Fig. 6 group V versus group II). We found that CTL activity was

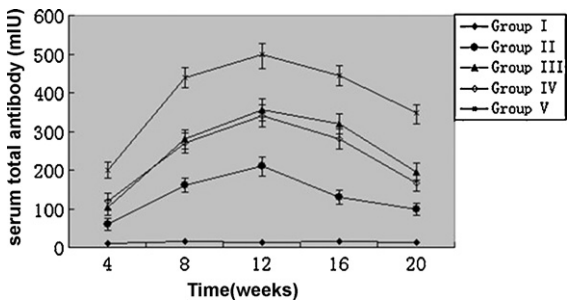

Fig. 4. Induction of HBsAg-specific total antibody in serum of C57BL/6 mice immunized i.m. or micro-needles with plasmid DNA encoding HBsAg. At the indicated times post-immunization, HBsAg-specific total antibody was analyzed in serum form individual mice in each group. Titres were determined as outlined in methods. Data are presented as mean ± S.D., where $n = 10$.

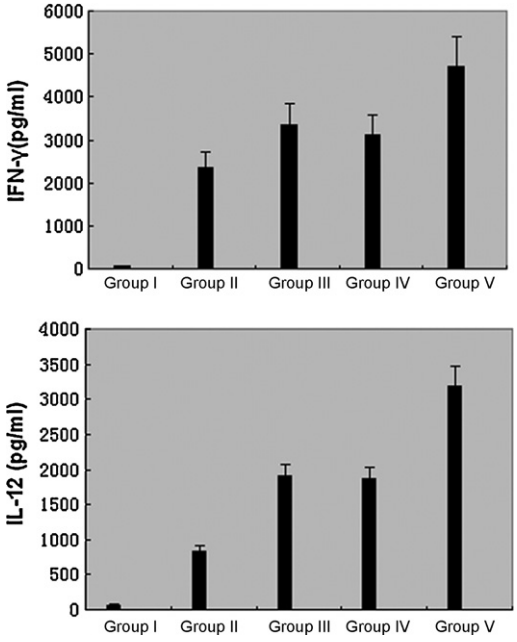

Fig. 5. HBsAg-specific cytokine IL-12 and IFN-γ production in splenocytes were obtained from mice from each group at 3 week after the final immunizations with different regimens described in Table 1. Pooled splenocytes of each group were stimulated with purified rHBsAg (10 μl/ml). The supernatants were harvested after 24 h (IL-12) and 72 h interferon γ (IFN-γ) and assayed the production of IFN-γ, IL-12. Values are from one representative experiment of three performed and are presented as the mean concentrations of triplicate wells ± S.D., where $n = 5$.

HBsAg-specific. Immunization with pVAX did not induce HBsAg-specific lysis. In addition, CTL activity was not observed in any group of mice when parental CT26 cells were used as target cells (data not shown). The effector cells that lysed HBsAg-expressing transfectants were eliminated by depletion of $CD8^+$, but not $CD4^+$, lymphocytes (Fig. 6). These results indicated that CTL activity was mediated by cells expressing a $CD4^-CD8^+$ surface phenotype. All experiments were performed three times, with similar results.

3.7. Protection of mice from CT/26 challenge

To determine whether the augmented immune response can result in protection *in vivo*, the immunoprotection in different formulations against challenge of transplanted HBsAg-expressing CT26/S was examined. As shown in Kaplan–Meier's curve (Fig. 7),

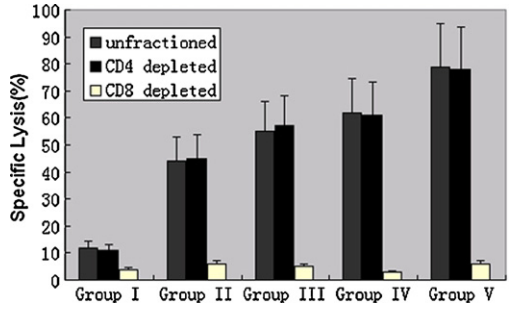

Fig. 6. HBsAg-specific CTL response of different vaccine regimens in mice. Splenocytes obtained from five mice per group at week 3 after third immunization were pooled and maintained in RPMI1640, 5×10^{-5} M 2-mercaptoenthanol and 10% fetal bovine serum. Responder cells (2.0×10^7) were re-stimulated *in vitro* with CT26/S cell (1.0×10^6) at 37 °C. After 1 week *in vitro* culture, the specific cytolytic reactivity of unfractionated, $CD8^+$-depleted or $CD4^+$-depleted responder as effector cells was tested. The E:T ratio is 100:1 in all of these assays. Values of specific lysis are from one representative experiment of three performed and are presented as the mean specific lysis of triplicate cultures ± S.D., where $n = 5$.

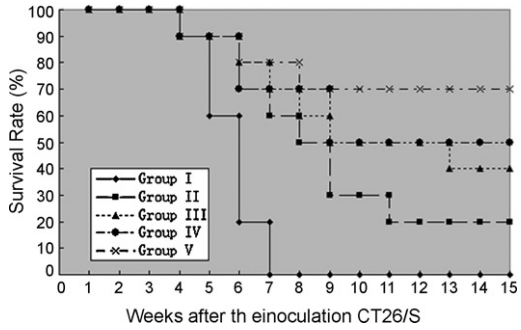

Fig. 7. Protection against challenge of CT26/S cell expressing HBsAg. 2.0×10^6 cells were transplanted in 10 mice of each group after the immunization with different formulations. The vitality of individual mouse in each group was monitored for 15 weeks post-immunization. The survival rate and time were shown through Kaplan–Meier's curve.

mice immunized with pVAX1 did not show any inhibitive effects on tumor formation, while mice immunized with pVAX(S) by i.m. or micro-needles alone showed weak protection efficacy, resulting a final survival rate of 20% or 40% at week 20. However, mice immunized with pVAX(S) adjuvant with pFlt3L by micro-needles displayed a strong inhibition on tumor formation and a remarkable improvement in final survival rate 70% (significantly higher compared with the groups of mice immunized by i.m., $P < 0.01$ by ANOVA analyses).

4. Discussion

Hepatitis B poses a serious worldwide problem. Lack of efficient antiviral treatment against HBV makes the development of a vaccine highly desirable. Current antigen-based vaccines have been widely used but there are disabilities to induce cellular immunity. There are many increasing evidences showing that ideal vaccine should be capable of eliciting both strong humoral and cellular immune response, especially Th1 and CTL responses [18,20]. DNA vaccine has it own advantages in eliciting strong humoral and cellular immune response. However, i.m. administered naked DNA vaccine has little immunogenicity for its difficult been taken up and expressed by muscular cells.

In the present study, we show that the DNA vaccine adjuvant with pFlt3L inoculated by micro-needles (Group V). Not only elicited significantly stronger CTL activities, but also induced the highest level of IFN-γ, IL-12 secretion compared with other fabrication described in Table 1. IFN-γ mediates the killing of organisms responsible for a variety of intracellular infections. It was reported that a functional Th1 response was crucially dependent on the cytokine, IL-12. Additional IL-12 plays a great role in cellular immunity eliminating HBV. Therefore we deem micro-needles combining with Flt3L resulted in stronger Th-1 type cellular immune response. So far, there is on satisfactory *in vivo* hepatitis B model, it is difficult to evaluate the protective efficacy against HBV elicited by immunization. We employed an HBsAg-expressing CT26/S model, which could partly represent HBV *in vivo* infection, to assess protective efficacy elicited by the immunization with different formulations. Our study shows that the DNA vaccine adjuvant with pFlt3L inoculated by micro-needles confers better protection.

The exact mechanism behind the optimal immunization regimens needs further active investigation. Intramuscular inoculation has been widely used for DNA immunization. Effective immune responses to intramuscular injection were reported in some cases [21,22]. Following intramuscular inoculation with a plasmid DNA virus, the DNA is taken up by the muscle cells, inducing antigen expression in transfected muscle cells. These transfected cells serve as APCs that then elicit immune response. However, although mus-

cle cell express MHC class I molecules, there is no detectable level of co-stimulatory molecules (CD80 and CD86) expression. These molecules are critical for T-cell priming. Therefore, the transfected muscle cells may not be as efficient as the professional APCs for antigen presentation [23,24]. There is overwhelming evidence suggesting that myeloid APCs, but not somatic cells, directly induce immune response after DNA vaccination. So many strategies have been introduced to target the DNA vaccine to APC to develop more efficient regimens of DNA vaccine [25–27]. In this study, we employ micro-needles to inoculate DNA vaccine to epidermis, where containing an abundance of antigen-presenting cells. Additional, adjuvant Flt3L is a potent DC-specific growth factor that has been reported to expand and to mature DCs in both mice and humans [9,28,29]. Therefore, the optimal site for vaccine delivery would be at a site with an abundance of mature APCs. Our experiments showed that inoculation with a DNA vaccine by micro-needles, with pFlt3L as an adjuvant, induced a stronger immune response than the other regimens. These data provide direct evidence that micro-needles and adjuvant pFlt3L facilitated DNA delivery to and uptake by professional APCs, such as monocytes and dendritic cell.

Our results indicated that the DNA vaccine delivery by micro-needles, with pFlt3L as an adjuvant, augmented the immune response necessary for development of protective immunity to HBV. These studies provide a strategy for improvement of immunization against HBV and many other pathogens. Our study may aid in future preventive and therapeutic designs for more effective methods of vaccination against HBV in humans.

Acknowledgements

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