Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 76 (2007) 140–149
Antiviral Research
www.elsevier.com/locate/antiviral

Coimmunization with RANTES plasmid polarized Th1 immune response against hepatitis B virus envelope via recruitment of dendritic cells

Ke Ma [a,b,1], Wei Xu [a,b,1], Xianan Shao [a,b], YanYue [a,b], Linkun Hu [a], Huanbin Xu [a,b], Zhigang Yuan [a,b], Xiujuan Zheng [a,b], Sidong Xiong [a,b,c,*]

[a] *Institute for ImmunoBiology and Department of Immunology, Shanghai Medical College of Fudan University, 138, Yi Xue Yuan Road, Shanghai 200032, PR China*
[b] *Key Laboratory of Molecular Medicine of the Ministry of Education, Shanghai 200032, People's Republic of China*
[c] *Immunology Division, E-Institutes of Shanghai Universities, Shanghai 200032, People's Republic of China*

Received 17 February 2007; accepted 19 June 2007

Abstract

Induction of T help cell type 1 (Th1) response seems to be a prerequisite of HBV clearance. DNA vaccines have shown its potential to elicit Th1-biased immune response. However, its immunogenicity needs to be improved. Regulated upon activation normal T cell expressed and secreted (RANTES) is an inflammatory chemokine that promotes the accumulation and activation of CD4+, CD8+ T cells, and dendritic cells (DCs), which would favor antiviral immunity. In this study, the efficacy of a DNA vaccine encoding hepatitis B virus (HBV) preS2 plus S protein was enhanced through co-injection of a plasmid encoding RANTES in a BALB/c model. Co-injection of RANTES gene resulted in a moderate increase in the HBV specific humoral and cellular immune responses and a significant increase following an HBsAg booster vaccination compared to DNA encoding HBsAg alone. This enhancement was due to an enrichment of DCs in the draining lymph node and an up-regulation of DCs maturation by RANTES. More importantly, RANTES polarized the specific immunity towards a dominant Th1 profile and even converted an established Th2 response to a Th1 phenotype. Our study suggested the feasibility of using a plasmid-encoded RANTES as a modulatory Th1 adjuvant in genetic vaccination.

Keywords: HBV; DNA vaccine; RANTES; DC; Th1

1. Introduction

Persistent hepatitis B virus (HBV) infection represents a major public health concern worldwide, with two billion people affected and an estimated 400 million people chronically infected which has a great propensity to progress to liver cirrhosis and hepatocellular carcinoma (HCC) (Michel and Mancini-Bourgine, 2005).

Recovery from acute HBV infection requires induction of both strong humoral and multispecific cellular immune responses (Mizukoshi et al., 2004). Patients with chronic HBV infection but with remission or with a self-limiting disease develop a vigorous CTL and a strong type-1 T helper (Th1) cell cytokine response represented by predominant production of TNFα and IFNγ. However, weak and limited CTL response is observed in chronic HBV carriers demonstrating the significance of Th1-biased immune response for HBV clearance (Beckebaum et al., 2002). Therefore strategies aimed to stimulate Th1 immunity would be very critical for the design of HBV vaccine.

DNA vaccination represents an attractive immunization strategy against infectious diseases since its discovery in 1990. The induction of CTLs, due to the *de novo* synthesis of Ag *in vivo*, makes DNA vaccines as effective as attenuated virus vaccines for treating chronic HBV infection (Davis and McCluskie, 1999). The CpG residues in the bacterial backbone make DNA vaccine prone to polarize Th1 immune response (Sato et al., 1996). Therefore DNA-based vaccine appears as a promising approach for prophylaxis and therapy of hepatitis B (Michel and Loirat, 2001; Thermet et al., 2003).

However, a major problem in developing DNA vaccines is their limited immunogenicity, which is highly dependent on function of dendritic cells (DCs). DCs are transfected directly by DNA or cross-primed indirectly by the antigen secreted from the transfected muscle cells (Doe et al., 1996) and then

* Corresponding author. Tel.: +86 21 54237324; fax: +86 21 54237749.
E-mail address: sdxiongfd@126.com (S. Xiong).
[1] These authors contribute equally to this work.

0166-3542/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2007.06.012

responsible for CD4+ or CD8+ T cell stimulation. Since the amounts of DCs residing in the muscle are really small, the chance DCs are transfected by the antigen is relatively very little. Efficiency of DNA vaccine may therefore be enhanced using DCs-intensifying strategies (Hung et al., 2006). Cytokines (Shimizu et al., 1998) and growth factors (Kwon and Park, 2002; Nayak et al., 2006) were widely used to increase the numbers or maturation of DCs. IgG Fc fragment (You et al., 2001) and heat shock protein (HSP) (Hauser et al., 2004) targeted antigens to DCs. Bclx was used to prolong DC life (Kim et al., 2003a,b) which contributed to enhanced CD8+ T and Th1 response. Regional recruitment of APCs represents another promising DC-intensifying strategy (Sang et al., 2003). Chemokines subsets, which attract immune cells, are ideal candidates for recruitment of DCs to the DNA injection site.

Regulated on activation normal T cell expressed and secreted (RANTES) is a member of the consecutive cysteines (CC)-chemokines that promotes the migration and activation of several types of leukocytes expressing CC-chemokine receptors 1 (CCR1), CCR3, and CCR5. It is able to recruit unstimulated CD4+ memory T cells, stimulated CD4+ and CD8+ T cells, natural killer cells, and immature DCs (Appay and Rowland-Jones, 2001; O'Neill et al., 2004). RANTES was effective to stimulate CD4+ T cell response (Shimizu et al., 2007; Sin et al., 2000a,b) and enhance HIV-specific Th1 responses and T-cell cytotoxicity in rodent and monkey models (Xin et al., 1999; Waterman et al., 2004,). In addition, RANTES increased the specific IgG production (Kim et al., 2003a,b). It also exhibited a proinflammatory effect by increasing the numbers of local infiltrating monocytes, macrophages, and neutrophils (Braciak et al., 1996). In particular, RANTES appears to be one of the main chemokines participating in the recruitment and maintenance of DC at the inflammatory site (Foti et al., 1999). Taken together, these data suggest RANTES as a potent monocyte chemoattractant and a Th1 adjuvant. We hypothesize that RANTES may act to enhance the Th1 immune response induced in the context of an HBV DNA vaccine.

To study the adjuvant effect of RANTES on a DNA vaccine encoding the middle envelope protein (PreS + S) of HBV, plasmid encoding PreS + S or RANTES was constructed separately and evaluated their expression efficiency *in vitro* and *in vivo*. The biological activity of RANTES was examined by a chemotaxis assay. Co-injection of HBsAg and RANTES gene into BALB/c mice was performed to see if RANTES could enhance the HBsAg-specific humoral and cellular immune response. A bicistronic plasmid coexpressing the two proteins was used to compare the effects.

2. Materials and methods

2.1. Plasmids construction

The RANTES gene was amplified from murine genomic DNA using a forward primer (5′-CG GGATCC GCCACC ATGAAGATCTCT-3′) engineered with a BamHI site upstream of the GOZAK sequence and the ATG, and a reverse primer (5′-C GAATTC CTAGCTCATCTCCAAATAGTT-3′) engineered with an EcoRI site downstream of the stop codon. The PCR fragment was subcloned into the pVAX1 vector (invitrogen, USA) to generate pRANTES. The middle envelope genes of HBV were amplified by PCR from plasmid pEHH, which contains full-length of HBV genome, using primers as 5′-C GAATTC GCCACC ATGCAGTGGAAC-3′ and 5′-T GGGCCC TTAAATGTATACCCAAAGACA-3′ both engineered with EcoRI sites and cloned into pVAX1 to generate pPreS2/S. The gene segment comprising CMV promoter, RANTES and polyA sequence was PCR-amplified from pRANTES and subcloned into the MluI site of pPreS2/S resulting in a bicistronic plasmid designated as pRBpPSB. Plasmid DNA was purified using EndoFree plasmid mega kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

2.2. DOT-immunoblot assay

C_2C_{12} (ATCC: CRL-1772) murine skeletal muscle cells was cultured in Dulbecco's modified Eagle's medium or RMPI 1640 supplemented with 10% fetal bovine serum (FBS). After DNA transfection using lipofectamine (Gibco BRL, USA) according to the protocol of manufacturer, protein expression in the supernatant was detected using a dot immunoblot assay according to a standard protocol (Loi et al., 1997). Twenty microliters of cell culture supernatants were spotted onto a 0.22-μm nitrocellulose membrane (Amersham, USA). The membrane was air dried at room temperature and fixed at 37 °C for 60 min. After washing, it was incubated for 60 min in a blocking solution containing 0.3% bovine serum albumin (BSA) in 0.1 M Tris buffer (pH 7.4) then was incubated overnight with a rat antimouse RANTES mAb (R and D systems, USA) or a mouse antihuman HBsAg mAb (Chemicon, USA), followed by a biotinylated secondary antibody (Dako, USA) for 30 min. The membrane was then transferred to a solution of streptavidin-biotin-peroxidase complex (Vector, USA) for 30 min and revealed by DAB substrate. The spot was taken as photographs, scanned into computer and adjusted for equal brightness and contrast using PhotoShop.

2.3. Immunohistochemical analysis of the muscle

Hundred micrograms of RANTES or PreS2/S plasmid was injected into the quadriceps muscles of mice, which were removed quickly before making cryosections. Seven days later, following acetone and normal serum treating, sections were incubated with rat antimouse RANTES mAb (R and D systems, USA) or a mouse antihuman HBsAg mAb (Chemicon, USA) overnight at 4 °C. Following three times of washing in PBS, sections were incubated for 1 h with biotinylated secondary antibody (Dako, USA) at 37 °C then for 30 min with streptavidin-biotin-peroxidase complex (Vector, USA) before revealed by DAB.

2.4. DC culture and chemotaxis assay

Immature and mature DCs were cultured by standard method. Briefly, murine bone marrow cells were extracted and resus-

pended as 1×10^6/ml in RMPI 1640 plus 10% FBS. For the generation of immature DCs, BM cells were incubated with 4 ng/ml GM–CSF for 48 h then with 4 ng/ml GM–CSF for another 72 h of incubation. To stimulate mature DC, 10 ng/ml GM–CSF and then 10 ng/ml GM–CSF plus 200 ng/ml TNFα were used. For the chemotaxis assay, 27 μl of preheated RANTES-transfected supernatant and were added into the lower chemotaxis chamber (Neuroprobe, USA); while 45 μl of DCs pretreated with or without anti-RANTES mAb (R and D Systems, USA) was placed into the upper chamber. After 4 h incubation at 37 °C, migrated cells in the lower chamber were stained and counted.

2.5. Mice immunization

Female BALB/c, 6–8 weeks old, was obtained from the center of experimental animal, Fudan University (Shanghai, China). All animals were housed in the pathogen-free mouse colony and all animal experiments were performed according to the guidelines for the care and use of medical laboratory animals (Ministry of Health PR China, 1998) and the guidelines of the laboratory animal ethical commission of Fudan University. For DNA injection, groups of BALB/c ($n = 5$) were 24 h before preinjected with 100 μl of 0.25% bupavokein (Sigma, St. Louis, MO) into the tibialis anterior muscle to induce muscle regeneration following cardiotoxin-induced degeneration. Then the same muscle was co-injected with 100 μg pPreS2/S DNA plus 100 μg pRANTES DNA diluted in 100 μl PBS. Control mice were immunized with 100 μg pVAX1 DNA. For protein boosting, 2 μg of yeast-derived HBsAg (SABC, China) was given subcutaneously 12 weeks later. Serum samples from mice were collected by retro-orbital bleeding.

2.6. ELISA measurements of HBsAg-specific antibodies

HBsAg-specific antibody was measured by ELISA assay. Microtiter plates were coated with 5 μg purified HBsAg protein in 100 μl PBS for each well. After blocking with 5% NBS and 5% goat serum in PBS-Tween (0.05%) for 1 h, two-fold serially diluted serum samples were added and incubated for 1 h at 37 °C then with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotech, USA). After color developing by OPD, the reaction was stopped by 2 N H_2SO_4, and absorbance at 492 nm was measured in a microplated reader (BioLab, USA).

2.7. Lymphoproliferative assay

CD4+ T cell proliferation was evaluated following *in vitro* stimulation with 10 μg/ml HBsAg. Splenocytes from immunized mice were isolated and were used as responders. Concanavalin A was used as a positive stimulatory reagent control. The cultures were incubated for 72 h at 37 °C in 5% CO_2, pulsed with 3.7×10^4 Bq [^{3}H]-thymidine (Amersham, USA) per well during the last 18 h of incubation, and then were harvested. [^{3}H]-thymidine uptake was determined by standard liquidscintillation.

2.8. Cytotoxicity analysis

A standard chromium release assay was used to monitor CTL activity. Briefly, 5×10^6 SP2/0 (ATCC: CRL-1646) cells were transiently transfected with pPreS2/S DNA and labeled with 7.4×10^6 Bq ^{51}Cr (Amersham, USA) for 90 min and used as target cells. They were mixed at appropriate ratios with restimulated effectors in duplicate and incubated at 37 °C for 4 h. Supernatants were harvested and radioactivity was counted in a gamma counter (LKB, Gaithersburg, MD). Each animal was analyzed independently. Percent-specific lysis was calculated using the equation: $100 \times$ [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Results are presented as HBsAg-specific lysis, calculated by subtracting the specific lysis of control SP2/0 target cells from the specific lysis of the pPreS2/S DNA-transfected SP2/0 target cells at the same E:T ratio. Spontaneous release was determined by incubating labeled target cells with RPMI 1640 and ranged between 10 and 15% in various experiments. Maximum release was determined by lysing the target cells with 5% SDS.

2.9. Flow cytometry

The inguinal LNs and the splenocytes were harvested and single cell suspensions were prepared by mechanical dissociation. One milliliter aliquots of the cells at a concentration of 10^6 cells/ml in PBS (1% BSA and 0.05% NaN3) were labeled with 1 μg/ml anti-CD11c-FITC and anti-CD86-PE or anti-I-A^d-PE conjugated antibodies (BD Biosciences PharMingen, San Diego, CA) for 30 min. Cells were washed, fixed with 2% paraformaldehyde, and acquired with a FACScan Plus (Becton Dickinson, Boston, MA). All data represent cells that fall within the lymphocyte gate determined by forward and 90-degree light scatter. Fifty thousand events were acquired and the frequency of DC subsets determined with AttractorsTM 3.0 software.

2.10. Statistical analysis

Data are shown as means and standard deviations; Statistical analyses of data were performed using the GraphPad Prism (Version 4.0) statistical program. Means were compared using the Student's two-sample *t*-test. A probability of less than 0.05 was taken as significant.

3. Results

3.1. Cloning of HBV preS2/S and RANTES gene into pVAX1 vector

As shown in Fig. 1, gene coding for the middle envelop protein (PreS2 plus S) of HBV or RANTES was placed under the transcriptional control of cytomegalovirus promoter/enhancer in the eukaryotic expression vectors pVAX1, resulting in plasmids designated as pPreS2/S and pRANTES, respectively. In the bicistronic vector, pRBpPSB, HBsAg and RANTES genes were under discrete transcriptional control.

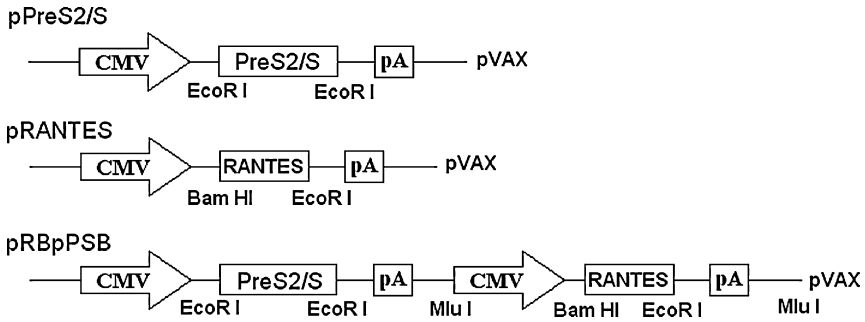


Fig. 1. Schematic diagram of expression vectors for HBsAg and RANTES. CMV, cytomegalovirus promoter/enhancer; BGH, bovine growth hormone.

3.2. *In vitro and in vivo expression of RANTES and HBsAg proteins*

To verify the transcriptional efficiency of pPreS2/S and pRANTES *in vitro*, a lipofectamin-aided transfection assay was conducted on C_2C_{12} skeletal muscle cells. The presence of HBsAg or RANTES protein in the culture supernatant was analyzed by a DOT-EIA assay. As shown in Fig. 2A and C, the expression of HBsAg and RANTES was detectable as soon as 24 h and peaked at 24 h posttransfection.

To measure the *in vivo* expression, BALB/c mice were injected intramuscularly with 100 μg pPreS2/S or pRANTES DNA, and the tibialis anterior muscle was sectioned seven days later and subjected to immunochemistry examination. HBsAg and RANTES staining was only visualized in muscles injected with pPreS2/S or pRANTES (Fig. 2B and D), not in the mock-injected muscle. Both of the protein expression could be observed on the first day (data not shown), lasting at least seven days after injection.

3.3. *Co-injection of RANTES with HBsAg DNA increased anti-HBsAg antibody induction*

In order to analyze the effect of RANTES coexpression along with HBsAg DNA on HBV-specific humoral immune response, BALB/c mice were inoculated with 100 μg DNA of pPreS2/S plus 100 μg pRANTES. Serum IgG was analyzed by ELISA assay. Mice injected with pPreS2/S plus pRANTES produced long-lasting serum anti-HBsAg IgG after Week 4, the titer of which being significantly higher than that of mice receiving pPreS2/S alone from Week 12 (1:1520 vs. 1:720, $p = 0.0158$), peaking at Week 18 (1:3880 vs. 1:360, $p = 0.0035$) and maintained that level at least to Week 22 (Fig. 3A).

The effect of a bicistronic vector (pRBpPSB, Fig. 1) coexpressing PreS2/S and RANTES was compared with the co-injection strategy. However, pRBpPSB immunization could only slightly enhance the IgG titer (peak titer 1:900) while no significant difference ($p = 0.0792$) was seen compared with pPreS2/S immunization group, although a significant difference existed between pRBpPSB and pVAX control group ($p < 0.001$).

In another parallel experiment, mice immunized with DNA vaccine at Week 0 were boosted with HBsAg proteins at Week 12 to measure the memory Ab responses. pPreS2/S plus pRANTES co-injection showed a robust memory responses which produced significant higher IgG titer than the pPreS2/S DNA vaccine alone from Week 16 (1:13,600 vs. 1:2200, $p = 0.0087$, Fig. 3B), Even at Week 24 (ten weeks after boosting), the titer generated by co-injection was significant higher than that by the one DNA-injection(1:60,000 vs. 1:12,000, $p = 0.0188$).The HBsAg booster immunization induced HBsAg-specific IgG response in pVAX1 and pRANTES control group as well, but the IgG titer was much lower, only 1:2400 and 1:4600 separately. The

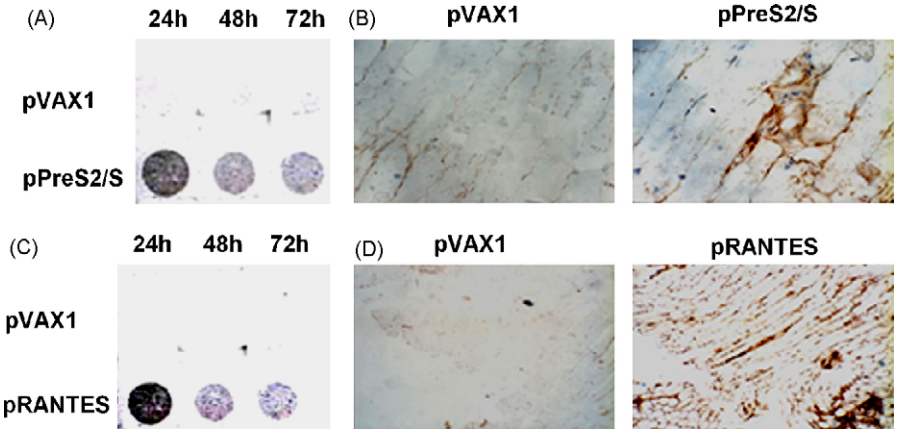


Fig. 2. *In vitro* and *in vivo* expression of pPreS2/S and pRANTES *in vitro* expression of HBsAg or RANTES was evaluated by a dot-immunoblot assay 24–72 h following transfection of murine C_2C_{12} cells with Lipofectamine-aided plasmid. *In vivo*, 100 μg pVAX, pPreS2/S or pRANTES DNA was injected to mice, the quadriceps muscles was removed and made cryosections and then stained with rat anti-mouse RANTES or a mouse anti-human HBsAg mAb. Fig. 2B and D showed the immunohistochemical analysis of the muscle tissue seven days following DNA injection (magnification: ×200).

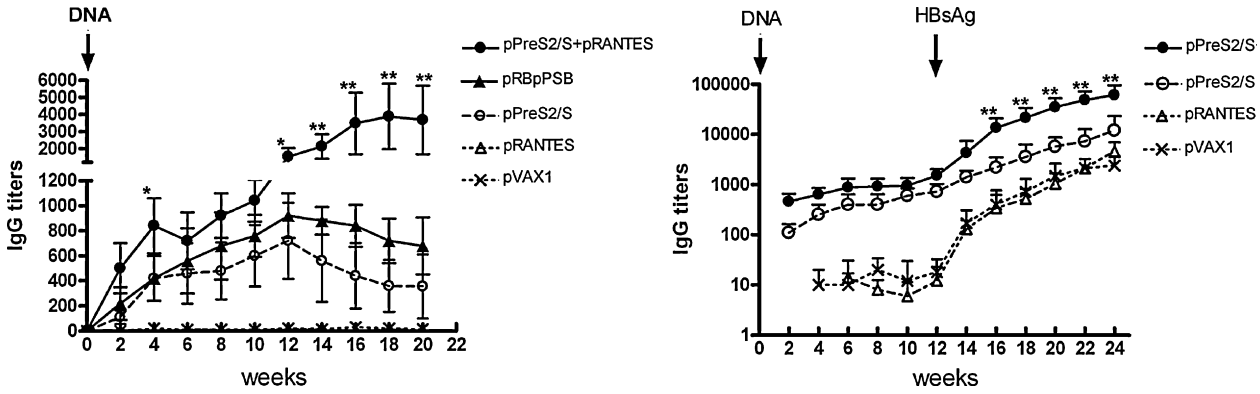


Fig. 3. HBsAg-specific serum IgG production after coimmunization with HBsAg and RANTES plasmid. BALB/c mice were i.m. immunized with a single injection of 100 μg of pPreS2/S plus pRANTES, pRBpPSB, pPreS2/S, pRANTES or pVAX1. Presence of IgG in the sera was examined by ELISA assay before (A) or after HBsAg boosting (B). HBsAg protein was subcutaneously injected 12 weeks after the DNA priming. Data were presented as mean ± S.D. representing five animals for each group. Asterisk(s) indicate statistically significant differences, that is, $p < 0.05$ (*) or $p < 0.01$ (**), relative to Ab titers of mice immunized with pPreS2/S alone.

boosting results confirmed that co-injection of pRANTES plasmid could significantly increase the HBsAg-specific memory Ab production by pPreS2/S DNA vaccine.

3.4. Co-injection of RANTES gene augmented specific CD4+ T cell proliferation and CD8+ CTL response after booster immunization

To measure the effects of cotransfer of RANTES with HBsAg DNA on the T cell-mediated immune response, CD4+ Th cell proliferation and CD8+ T cell cytotoxicity were evaluated. RANTES co-injection slightly enhanced the proliferation of CD4+ T cells by a single pPreS2/S injection. While a significantly higher Th proliferation conferred by RANTES co-injection was observed following HBsAg boosting (Fig. 4A, $p < 0.05$).

Cytotoxicity assay also showed that, pRANTES co-injection slightly elevated the specific CTL cytotoxicity by pPreS2/S immunization. This enhancement was significant only after the booster immunization (23.5% vs. 13.2%, 10:1; $p < 0.05$,

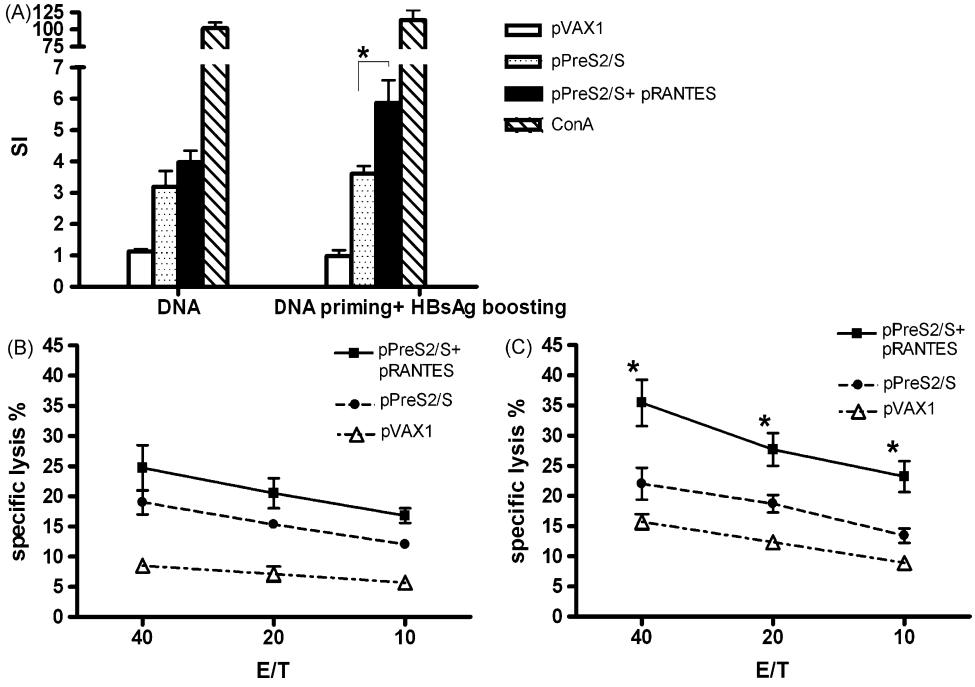


Fig. 4. T cell immune response induced by co-injection of RANTES DNA with HBsAg DNA as measured by CD4+ T proliferation (A) and CTL activity (B). Four weeks after the DNA injection or HBsAg boosting, the splenocytes were isolated and incubated in the presence of the following stimulators and controls: 1640 medium (negative control), ConA (5 μg/ml, positive control), HBsAg (10 μg/ml). CTLs were prepared *in vitro* by stimulating splenocytes with 10 μg/ml HBsAg protein and 20 U/ml IL-2 for 48 h. pPreS2/S-sustained transfected SP2/0 was used as target cells. After 4 h incubation, the Cr^{51} in the supernatant was measured by γ-counter. Data are presented as the SI (experimental counts/spontaneous counts), where the spontaneous count wells are from the 1640-negative control wells. Values represent the mean of triplicate cultures and are representative of three independent experiments. *$p < 0.05$.

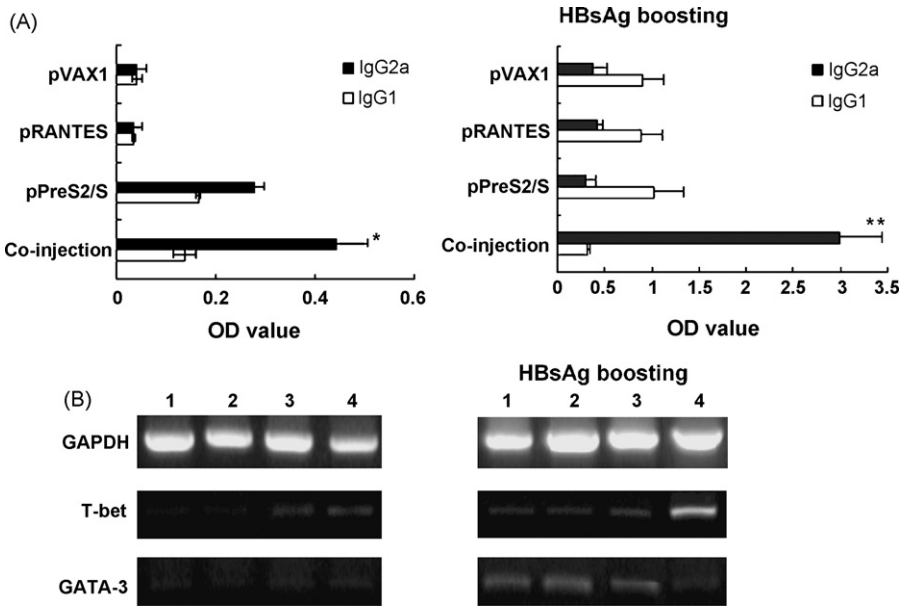


Fig. 5. RANTES gene coimmunization directed Th1 polarization as measured by IgG isotype (A) and Th-associated transcription factor expression (B). Four groups of mice were immunized with 100 μg of pVAX1 (Lane 1), pRANTES (Lane 2), pPreS2/S (Lane 3) or pPreS2/S +pRANTES (Lane 4), an HBsAg protein boosting was administered at Week 12, presence of HBV-specific serum IgG1 or IgG2a was evaluated by ELISA four weeks after the DNA priming or ten weeks after the boosting. Meanwhile, RT-PCR was performed to evaluate the mRNA expression level of Th transcriptional factor T-bet and GATA-3. Relative expression was calculated by dividing the band intensity of each CK product by that of GAPDH. *, $p < 0.05$; **, $p < 0.01$.

Fig. 4B). Altogether, RANTES DNA coimmunization moderately enhanced HBV-specific cellular immune response.

3.5. RANTES co-injection dominantly enhanced the Th1 polarization

Effects of RANTES on the Th immune deviation was assessed by evaluation of the serum Ab isotype profiles (reflecting Th1 or Th2 polarization) and Th-associated transcriptional factors expression at the fourth and 22nd weeks. As shown in Fig. 5A, co-injection of pRANTES significantly enhanced IgG2a production over that with pPreS2/S DNA vaccine alone (IgG2a/IgG1 ratio as 3.25 vs. 1.70, $p < 0.05$). The HBsAg booster generated a significant increase in the level of IgG2a while a decrease in IgG1 resulting in a significantly augmented IgG2a/IgG1 ratio (9.31 vs. 0.29, $p < 0.01$) (Fig. 5A right) suggesting that pRANTES dominantly oriented the Th1 polarization and could even convert the established Th2 polarization back to a Th1 phenotype.

Consistent with the above results, RANTES gene co-injection significantly enhanced Th1 transcriptional factor expression of T-bet especially after HBsAg boosting ($p < 0.01$). While the same boosting triggered a dominant expression of Th2 transcriptional factor GATA-3 in the pVAX and pPreS2/S treated mice (Fig. 5B) confirming RANTES as a good Th1 adjuvant.

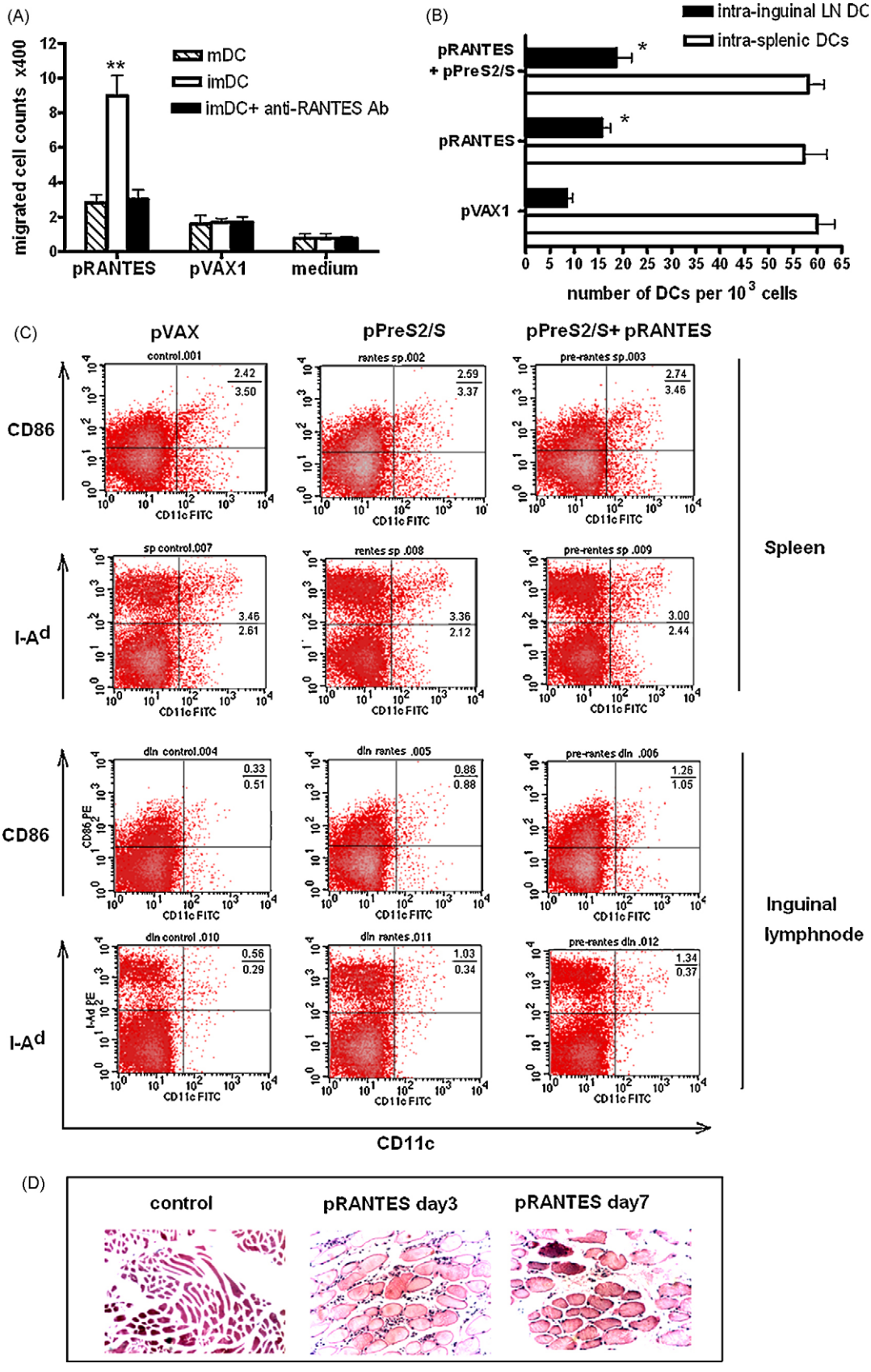
3.6. RANTES co-injection promoted the migration of DCs to the draining lymphoid node with elevated maturation-associated phenotype

We anticipated that the immune promotion by RANTES might be implemented through DC intensification. To assess this hypothesis, first, effects of RANTES on DC chemotaxis were evaluated *in vitro* by enumeration of migrated DCs in response to the RANTES. It found that RANTES was only attractive for imDCs but not mDCs, and this chemotaxis was RANTES-specific since anti-RANTES mAb totally blocked this activity (Fig. 6A).

The effect of RANTES on DC accumulation in the draining LN and the spleen was then examined by flow cytometry. Following pRANTES injection, a significant increase in the number of CD11c+ DCs was observed in the inguinal draining lymph node (IDL) compared to pVAX1 immunization (1.56% vs. 0.84%, $p < 0.05$, Fig. 6B and C), but not in the spleen (6.00% vs. 5.72%, $p > 0.05$). Co-injection of pRANTES and pPreS2/S moderately enhanced the DC recruitment (1.86%) into the IDL.

We next assessed whether pRANTES could influence the maturation of local infiltrated DCs. Fig. 6C showed that the pRANTES injection significantly increased the expression of maturation-associated phenotypic markers (CD86, I-A^d) on surface of DCs, holding 49.4% CD86+ DCs and 75.2% I-A^{d+} DCs within the total CD11c+ DCs in the ILN, significantly higher than the 39.2% CD86+ and 65.8% I-A^{d+} DCs infiltration seen after pVAX immunization ($p < 0.05$, Fig. 6C), indicating RANTES enhanced both the migration and the maturation of DCs in the draining lymph node. Co-injection of pRANTES and pPreS2/S moderately enhanced the maturation of DCs, possessing 54.5% CD86+ and 78.3% I-A^{d+} DCs in the IDL.

To see the immune infiltration at the primary injection site, HE-staining of the injected quadriceps muscle revealed no immune infiltration after pVAX vaccination (Fig. 6D, left); while pRANTES injection induced a substantial higher level of inflammatory cell infiltration on Day 3 and Day 7 (Fig. 6D, middle


(A)
migrated cell counts x400
mDC
imDC
imDC+ anti-RANTES Ab
**
pRANTES
pVAX1
medium
(B)
intra-inguinal LN DC
intra-splenic DCs
pRANTES + pPreS2/S
pRANTES
pVAX1
*
*
number of DCs per 10^3 cells
(C)
pVAX
pPreS2/S
pPreS2/S+ pRANTES
CD86
I-Ad
Spleen
Inguinal lymphnode
CD11c
CD11c FITC
CD86 PE
I-Ad PE
control.001
rantes sp.002
pre-rantes sp.003
sp control.007
rentes sp .008
pre-rentes sp .009
dln control.004
dln rantes .005
pre-rantes dln .006
dln control .010
dln rantes .011
pre-rantes dln .012
(D)
control
pRANTES day3
pRANTES day7

and right) indicating a considerable amount of DCs have been recruited into the DNA injection site.

All these results indicated that *in situ* expressed RANTES could efficiently chemoattract imDCs to the DNA injection site for the antigen uptake, leading to more matured DCs enrichment in the DLN, where they could stimulate HBsAg-specific T cell and B cell immune response more efficiently.

4. Discussion

We reported here that administration of a RANTES DNA by i.m. injection could induce more imDCs recruitment to the injection site and subsequent into the inguinal draining lymph node where they acquired elevated maturation phenotype (I-A^d and CD86), leading to an enhanced immune response to a HBV middle envelope plasmid vaccine as shown by both humoral and cellular immune assays.

However, the bicistronic vector (pRBpPSB, Fig. 1) coexpressing PreS2/S and RANTES failed to display the adjuvant effect of RANTES since one injection of pRBpPSB slightly increased HBV-specific IgG compared with pPreS2/S immunization and had no effect on the T cell response. The possible reason is that the two CMV promoters within one vector compete with each other, thus impairing the expression efficiency of RANTES and HBsAg.

As we have known, immunity to infection is controlled by distinct Th1 and Th2 subpopulations of T cells and their balance (McGuirk and Mills, 2002). As an effective DNA vaccine, the induction of Th1-type response is important for prevention of HBV infection (Mizukoshi et al., 2004). DCs play a critical role in the stimulation of Th1 immune response by DNA vaccines (Gurunathan et al., 2000), and their potential is not fully utilized in the DNA vaccine setting since they take up only a minor fraction of the injected DNA in the muscle. Therefore DCs-intensifying approaches have been utilized including growth-facilitative, function stimulating, or life-prolonging strategies (Haddad et al., 2000; Ara et al., 2001; Hung et al., 2006; Eo et al., 2001). We employed a natural DC-chemoattractant molecule, RANTES, to recruit more DCs to the antigen expression site to facilitate improved antigen-processing and presentation.

An important finding of this study is that we found RANTES dominantly polarized Th1 immune responses and could even convert the protein immunization-driven Th2 immunity to the Th1 profile as evidenced by predominant IgG2a production and T-bet expression. We reasoned that RANTES directed Th1 polarization via the recruitment and stimulation of DCs at the primary inflammatory site. It has been well documented that RANTES treatment (10–100 ng/ml) will induce DC's rapid expression of transcripts for the cytokines TNFα and IL-6 (Fischer et al., 2001) which are direct potent DC maturation stimuli (O'Neill et al., 2004). Therefore, following pRANTES immunization, the *in situ* expressed RANTES would attract more CCR1+/CCR5+ imDCs to the muscle (as demonstrated by Fig. 6D) and activate immature DC to secret inflammatory cytokine TNFα and IL-6. This autocrine secretion promotes their maturation by up-regulating the expression of costimulatory and MHC II molecules (Fig. 6C) and facilitates their movement into the inguinal draining lymphonode (Fig. 6B) where T cell activation and Th differentiation was initiated. Since RANTES has been reported to stimulate lymphocytes to secret Th1 cytokine (IL-2, IFNγ) (Sin et al., 2000a,b; Yoon and Eo, 2007) which would facilitate both DC1 and Th1 stimulation; RANTES could directly influence a variety of other CCR1+, CCR5+ cells predominantly associated with Th1 responses (Fukada et al., 2002); and our study also demonstrated that RANTES significantly up-regulated the expression of T-bet, a master transcription factor essential for Th1 development. Altogether, RANTES induces the recruitment and maturation of imDCs in the draining lymphonode, meanwhile promotes the Th1 cytokines secretion by a variety of immune cells and T-bet expression in T cells, which drives Th1 polarization.

Induction of Th1 immune responses renders the recipients more resistant to virus infection. Therefore various molecules, chemokines, and cytokines have been reported to orient naïve CD4+Th cell toward Th1 polarization including CpG,IL-2, IL-12, IL-8, IFNγ, CD40L, B7, C5b-9, HSP70, CD38 (Sin et al., 2000a,b; Bhatia et al., 2002; Kumar et al., 2007). However, in case of chronic viral infection, it seems very difficult to convert an established Th2 phenotype back to a Th1 phenotype. Our study indicated that RANTES gene coimmunization not only modulated a Th1 differentiation, more importantly could convert the strong Th2-biased immune response established by a protein booster back to a Th1 type indicating RANTES as a potential therapeutic Th1-modulating chemokine. This unique function is similar as a scavenger receptors-based APCs-targeting strategy, which modulated an established Th2 immunity to a nonallergic Th1 phenotype (Bhatia et al., 2002).

Several other reports also confirmed the Th1-immunomodulatory effect of RANTES. RANTES DNA was able to enhance Th1-mediated protective immunity against herpes simplex virus (HSV) (Sin et al., 2000a,b), to up-regulate the costimulatory molecules expression (CD28, CD40L, and CD80) on CD4+ T and APCs (Lillard et al., 2001) and enhanced CD8+T cell response (Kim et al., 2003a,b).

In our trial to use RANTES as a genetic adjuvant, we confirmed that the best vaccine effects would be achieved only

Fig. 6. RANTES attracted more DCs *in vitro* (A) and *in vivo* (B, C, D). (A) DC-chemotactic activity by RANTES was assayed using Transwell chambers. The culture supernatant of pVAX1 or pRANTES-transfected C_2C_{12} cells was added into the lower chemotaxis chamber, DCs pretreated with or without anti-RANTES Ab was placed into the upper chamber. After 4 h incubation at 37 °C, migrated cells in the lower chamber were stained and counted. **, $p < 0.01$ compared with pVAX group. (B) The frequency of CD11c+DC subsets in the inguinal LN and the spleen was determined by flow cytometry seven days after the administration of 100 μg pVAX, pRANTES, or pRANTES+ pPreS2/S. Data are presented as mean ± SEM (five mice per group). *, $p < 0.05$ compared with pVAX control. (C) The expression of CD86 and I-A^d, the maturation- associated marker of DC was also determined by flow cytometry. One representative out of three independent experiments is shown. (D) Histologic examination of the pRANTES-injected .muscle. The quadriceps muscles were isolated three and seven days after immunization, fixed in 10% buffered formalin. Thin sections were stained with hematoxylin and eosin (magnification: ×200).

when RANTES was delivered as a co-injection plasmid DNA with an equal amount (single injection of 100 μg) with the PreS2/S DNA. This finding was consistent with a recent work confirming that co-injection (i.m.) of Flt3L DNA (for DC expansion) with DNA vaccine was required to reveal the best adjuvant activity of the FLt3L (Nayak et al., 2006). Because there is a critical timeframe for the local DC expansion after RANTES injection (from Day 0 to Day 7, Fig. 6D) during which the expression of HBsAg antigens *in vivo* also increased and peaked on Day 7 (Fig. 2B), therefore co-injection of RANTES and the antigen DNA provided more possibility for expanded DC to recognize specific antigen at the primary injection site.

Taken together, our study demonstrated that co-injection of a chemokine, RANTES, in the form of a DNA vaccine, polarized Th1 immune response, and could cause a rebalancing of the immunity to a Th1 profile, which would benefit the protective efficacy against a virulent virus infection.

Acknowledgements

This work was supported in part by China national natural science foundation program (30400396, 30671952), National high technology research and development program of China (2007CB512401), program for outstanding medical academic leader (LJ06011) and U.S. *Fortdogen* grant.

References

Appay, V., Rowland-Jones, S.L., 2001. RANTES: a versatile and controversial chemokine. Trends. Immunol. 22, 83–87.

Ara, Y., Saito, T., Takagi, T., Hagiwara, E., Miyagi, Y., Sugiyama, M., Kawamoto, S., Ishii, N., Yoshida, T., Hanashi, D., Koshino, T., Okada, H., Okuda, K., 2001. Zymosan enhances the immune response to DNA vaccine for human immunodeficiency virus type-1 through the activation of complement system. Immunology 103, 98–105.

Beckebaum, S., Cicinnati, V.R., Gerken, G., 2002. DNA-based immunotherapy: potential for treatment of chronic viral hepatitis? Rev. Med. Virol. 12, 297–319.

Bhatia, S., Mukhopadhyay, S., Jarman, E., Hall, G., George, A., Basu, S.K., Rath, S., Lamb, J.R., Bal, V., 2002. Scavenger receptor-specific allergen delivery elicits IFN-gamma-dominated immunity and directs established TH2-dominated responses to a nonallergic phenotype. J. Allergy. Clin. Immunol. 109, 321–328.

Braciak, T.A., Bacon, K., Xing, Z., Torry, D.J., Graham, F.L., Schall, T.J., Richards, C.D., 1996. Overexpression of RANTES using a recombinant adenovirus vector induces the tissue-directed recruitment of monocytes to the lung. J. Immunol. 157, 5076–5084.

Davis, H.L., McCluskie, M.J., 1999. DNA vaccines for viral diseases. Microbes Infect. 1, 7–21.

Doe, B., Selby, M., Barnett, S., Baenziger, J., Walker, C.M., 1996. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. Proc. Natl. Acad. Sci. USA. 93, 8578–8583.

Eo, S.K., Lee, S., Kumaraguru, U., Barry, T., 2001. Rouse. Immunopotentiation of DNA vaccine against herpes simplex virus via co-delivery of plasmid DNA expressing CCR7 ligands. Vaccine 19, 4685–4693.

Fischer, F.R., Luo, Y., Luo, M., Santambrogio, L., Dorf, M.E., 2001. RANTES-induced chemokine cascade in dendritic cells. J. Immunol. 167, 1637–1643.

Foti, M., Granucci, F., Aggujaro, D., Liboi, E., Luini, W., Minardi, S., Mantovani, A., Sozzani, S., Ricciardi-Castagnoli, P., 1999. Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site. Int. Immunol. 11, 979–986.

Fukada, K., Sobao, Y., Tomiyama, H., Oka, S., Takiguchi, M., 2002. Functional expression of the chemokine receptor CCR5 on virus epitope-specific memory and effector CD8+ T cells. J. Immunol. 168, 2225–2232.

Gurunathan, S., Klinman, D.M., Seder, R.A., 2000. DNA vaccines: immunology, application, and optimization. Annu. Rev. Immunol. 18, 927–974.

Haddad, D., Ramprakash, J., Sedegah, M., Charoenvit, Y., Baumgartner, R., Kumar, S., Hoffman, S.L., Weiss, W.R., 2000. Plasmid vaccine expressing granulocyte-macrophage colony-stimulating factor attracts infiltrates including immature dendritic cells into injected muscles. J. Immunol. 165, 3772–3781.

Hauser, H., Shen, L., Gu, Q.L., Krueger, S., Chen, S.Y., 2004. Secretory heat-shock protein as a dendritic cell-targeting molecule: a new strategy to enhance the potency of genetic vaccines. Gene Ther. 11, 924–932.

Hung, C.F., Yang, M., Wu, T.C., 2006. Modifying professional antigen-presenting cells to enhance DNA vaccine potency. Methods Mol. Med. 127, 199–220.

Kim, S.J., Suh, D., Park, S.E., Park, J.S., Byun, H.M., Lee, C., Lee, S.Y., Kim, I., Oh, Y.K., 2003a. Enhanced immunogenicity of DNA fusion vaccine encoding secreted hepatitis B surface antigen and chemokine RANTES. Virology 314, 84–91.

Kim, T.W., Hung, C.F., Boyd, D., Juang, J., He, L., Kim, J.W., Hardwick, J.M., Wu, T.C., 2003b. Enhancing DNA vaccine potency by combining a strategy to prolong dendritic cell life with intracellular targeting strategies. J. Immunol. 171, 2970–2976.

Kwon, T.K., Park, J.W., 2002. Intramuscular co-injection of naked DNA encoding HBV core antigen and Flt3 ligand suppresses anti-HBc antibody response. Immunol. Lett. 81, 229–234.

Loi, P.K., Mcgraw, H.F., Tublitz, N.J., 1997. Peptide detection in single cells using a dot immunoblot assay. Peptides 18, 749–753.

Lillard, J.W., Boyaka, P.N., Taub, D.D., McGhee, J.R., 2001. RANTES potentiates antigen-specific mucosal immune responses. J. Immunol. 166, 162–169.

McGuirk, P., Mills, K.H., 2002. Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigmin immunity to infectious diseases. Trends Immunol. 23, 450–455.

Michel, M.L., Loirat, D., 2001. DNA vaccines for prophylactic or therapeutic immunization against hepatitis B. Intervirology 44, 78–87.

Michel, M.L., Mancini-Bourgine, M., 2005. Therapeutic vaccination against chronic hepatitis B virus infection. J. Clin. Virol. 34 (1), S108–S114.

Mizukoshi, E., Sidney, J., Livingston, B., Ghany, M., Hoofnagle, J.H., Sette, A., Rehermann, B., 2004. Cellular immune responses to the hepatitis B virus polymerase. J. Immunol. 173, 5863–5871.

Nayak, B.P., Sailaja, G., Jabbar, A.M., 2006. Augmenting the immunogenicity of DNA vaccines: role of plasmid-encoded Flt-3 ligand, as a molecular adjuvant in genetic vaccination. Virology 348, 277–288.

O'Neill, D.W., Adams, S., Bhardwaj, N., 2004. Manipulating dendritic cell biology for the active immunotherapy of cancer. Blood 104, 2235–2246.

Sang, H., Pisarev, V.M., Munger, C., Robinson, S., Chavez, J., Hatcher, L., Parajuli, P., Guo, Y., Talmadge, J.E., 2003. Regional, but not systemic recruitment/expansion of dendritic cells by a pluronic-formulated Flt3-ligand plasmid with vaccine adjuvant activity. Vaccine 21, 3019–3029.

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.D., Silverman, G.J., Lotz, M., Carson, D.A., Raz, E., 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 273, 352–354.

Shimizu, Y., Guidotti, L.G., Fowler, P., Chisari, F.V., 1998. Dendritic Cell Immunization Breaks Cytotoxic T Lymphocyte Tolerance in Hepatitis B Virus Transgenic Mice. J. Immunol. 161, 4520–4529.

Shimizu, Y., Inaba, K., Kaneyasu, K., Ibuki, K., Himeno, A., Okoba, M., Goto, Y., Hayami, M., Miura, T., Haga, T., 2007. A genetically engineered live-attenuated simian-human immunodeficiency virus that co-expresses the RANTES gene improves the magnitude of cellular immunity in rhesus macaques. Virology 361, 68–79.

Sin, J., Kim, J.J., Pachuk, C., Satishchandran, C., Weiner, D.B., 2000a. DNA vaccines encoding interleukin-8 and RANTES enhance antigen-specific Th1-type CD4(+) T-cell-mediated protective immunity against herpes simplex virus type 2 in vivo. J. Virol. 74, 11173–11180.

Sin, J.I., Kim, J.J., Pachuk, C., Satishchandran, C., Weiner, D.B., 2000b. DNA vaccines encoding interleukin-8 and RANTES enhance antigen-specific Th1-type CD4 T-cell-mediated protective immunity against herpes simplex virus type 2 in vivo. J. Virol. 74, 11173–11180.

Thermet, A., Rollier, C., Zoulim, F., Trepo, C., Cova, L., 2003. Progress in DNA vaccine for prophylaxis and therapy of hepatitis B. Vaccine 1, 659–662.

Waterman, P.M., Kitabwalla, M., Hatfield, G.S., Evans, P.S., Lu, Y., Tikhonov, I., Bryant, J.L., Pauza, C.D., 2004. Effects of virus burden and chemokine expression on immunity to SHIV in nonhuman primates. Viral. Immunol. 17, 545–557.

Xin, K.Q., Lu, Y., Hamajima, K., Fukushima, J., Yang, J., Inamura, K., Okuda, K., 1999. Immunization of RANTES expression plasmid with a DNA vaccine enhances HIV-1-specific immunity. Clin. Immunol. 92, 90–96.

Yoon, H.A., Eo, S.K., 2007. Differential polarization of immune responses by genetic cotransfer of chemokines changes the protective immunity of DNA vaccine against pseudorabies virus. Immunology 120, 182–191.

You, Z., Huang, X., Hester, J., Toh, H.C., Chen, S.Y., 2001. Targeting dendritic cells to enhance DNA vaccine potency. Cancer Res. 61, 3704–3711.