Enhancement of Antigen-specific Antibody and CD8$^+$ T Cell Responses by Codelivery of IL-12-encapsulated Microspheres in Protein and Peptide Vaccination

Su-Hyung Park[1], Jun Chang[2], Se-Hwan Yang[1], Hye-Ju Kim[1,3], Hyun-Hee Kwak[3], Byong-Moon Kim[3], Sung-Hee Lee[3] and Young Chul Sung[1]

[1]*Division of Molecular and Life Sciences, Postech Biotech Center, Pohang University of Science & Technology, Pohang,* [2]*College of Pharmacy, Ewha Womans University, Seoul,* [3]*Research Laboratories, Dong-A Pham. Co., Ltd., Yongin, Korea*

ABSTRACT

Background: Although IL-12 has been widely accepted to play a central role in the control of pathogen infection, the use of recombinant IL-12 (rIL-12) as a vaccine adjuvant has been known to be ineffective because of its rapid clearance in the body. **Methods:** To investigate the effect of sustained release of IL-12 *in vivo* in the peptide and protein vaccination models, rIL-12 was encapsulated into poly ($_{DL}$-lactic-co-glycolic acid) (PLGA). **Results:** We found that codelivery of IL-12-encapsulated microspheres (IL-12EM) could dramatically increase not only antibody responses, but also antigen-specific CD4$^+$ and CD8$^+$ T cell responses. Enhanced immune responses were shown to be correlated with protective immunity against influenza and respiratory syncytial virus (RSV) virus challenge. Interestingly, the enhancement of CD8$^+$ T cell response was not detectable when CD4$^+$ T cell knockout mice were subjected to vaccination, indicating that the enhancement of the CD8$^+$ T cell response by IL-12EM is dependent on CD4$^+$ T cell "help". **Conclusion:** Thus, IL-12EM could be applied as an adjuvant of protein and peptide vaccines to enhance protective immunity against virus infection. **(Immune Network 2007;7(4): 186-196)**

Key Words: Interleukin 12, PLGA microspheres, vaccine adjuvant, CTL response

Introduction

Cell-mediated immunity with cytotoxic T lymphocytes (CTL) immune response plays a key role in protection against pathogenic virus infection, but is barely induced by proteins. Although DNA and recombinant virus vaccines are known to efficiently induce Th1 and CTLs, protein and peptide vaccines are inefficient for inducing CD8$^+$ and CD4$^+$ T cell responses, respectively. Therefore, the advancement of good adjuvants promoting cell-mediated immunity is one of the major concerns for developing effective subunit vaccines against viral diseases.

IL-12 has been shown to have potent activity as an immune-activating agent for treatment of tumors and infectious diseases, and is also regarded as an adjuvant in prophylactic and therapeutic vaccines against viral diseases (1,20). However, the use of recombinant IL-12 protein *in vivo* as an adjuvant of protein or peptide vaccines was insufficient in improving antigen-specific T cell responses. Lindblad et al. reported that the addition of IL-12 into a TB subunit vaccine could amplify T cell response in a dose-dependent manner and pro-

Correspondence to: Young-Chul Sung, Division of Molecular and Life Sciences, Postech Biotech Center, Pohang University of Science & Technology, San 31, Hyoja-dong, Pohang 790-784, Korea (Tel) 82-54-279-2294, (Fax) 82-54-279-5544, (E-mail) ycsung@postech.ac.kr

This work was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A040013), supported by a grant from the KOSEF funded by the Korea government (MOST) (M10534050001-06N3405-00110), supported by a grant from SRC fund to IRC at University of Ulsan from the KOSEF & the Korea Ministry of Science & Technology, and partly supported by a grant from the NCRC program of the Ministry of Science & Technology and the Korea Science and Engineering Foundation through the Center for Cell Signaling & Drug Discovery Research at Ewha Womans University (R15-2006-020).

mote a protective immune response against a virulent challenge (30). However, no improvement was observed in terms of long-term efficacy, suggesting that rIL-12 may exert a transient effect (30). Similar results have been found by other investigators for subunit vaccines against L. major (15) and T. gondii (43). Regarding the action mechanism of IL-12, it was demonstrated that the persistence of IL-12 is crucial for maintenance and initiation of cell-mediated immunity (15).

Biodegradable polymeric microspheres as injectable depots for protein and peptide drugs have been extensively investigated for the past two decades (27) and used in humans for drug delivery (28), cancer chemotherapy (21) and vaccination with antigenic peptides (35). Although these delivery systems were initially developed for sustained delivery of low molecular weight therapeutics, advances in encapsulation technologies and protein stabilization have led to the successful encapsulation of a number of bioactive macromolecules, including immunostimulatory cytokines (36). The use of IL-12 encapsulated microspheres (IL-12EM) could achieve local and sustained expression of IL-12 to overcome the rapid clearance of IL-12 protein *in vivo*. As expected, the sustained release of IL-12 from the microspheres was superior to the injection of rIL-12 in the regression of primary tumor and prevention of metastatic spread to the lungs (12). However, the immunemodulating effect of IL-12EM is not yet clear in terms of induction of antigen-specific antibody and T cell responses.

In this study, we found that IL-12EM could significantly enhance $CD4^+$ and $CD8^+$ T cell responses, as well as antibody responses in protein and peptide vaccines; these responses are dependent on the $CD4^+$ T cells "help". In addition, the enhanced immune responses are closely associated with protection against RSV and influenza virus challenge. Thus, we propose that IL-12EM may be used as a T cell adjuvant for peptide and protein vaccines.

Materials and Methods

Mice. Specific pathogen-free female BALB/c, C57BL/6 and $CD4^+$ T cell knockout mice with 5～6 weeks of age were purchased from Japan SLC, Inc. (Shijuoka, Japan), maintained under barrier conditions in a BL-3 biohazard animal room at Pohang University of Science and Technology, and fed a sterile commercial mouse diet and water. All mice experiments were done under the approval and regulations of local animal experimentation ethics committee of Pohang University of Science and Technology.

Peptide synthesis. RSV M2-specific (H-2^d-restrcited, SYIGSINNI), HBsAg-specific (H-2^d-restricted, IPQSLPSWWTSL) and influenza HA-specific (H-2^d-restricted, IYSTVASSL) CTL epitopes were synthesized by PEPTRON (Daejeon, Republic of Korea). As possible H-2^b-restricted HA-specific CTL epitopes, nine 8～10 mer peptides (SQLKNNAKEI, SSFYRNLLWL, LVLLE NERTL, TGLRNIPSI, SNVKNLYEKV, KINYYWTL, QGEVNVTGVI, YGDSNPQKF and IKSHFANL) were synthesized on the basis of estimated half-lives of dissociation from H-2^b molecule using a peptide binding prediction algorithm (http://bimas.dcrt.nih.gov/molbio /hla_bind/).

IL-12-encapsulated microspheres. A water-in-oil-in-water (w/o/w) double-emulsion solvent evaporation technique (8,22) was used for encapsulation of cytokine IL-12. Briefly, 500 ul of aqueous solution of recombinant mouse IL-12 (50 ug) and bovine serum albumin (BSA, 12.5 mg) was emulsified in 1.2 ml of dichloromethane (DCM) containing 500 mg of polylactic-co-glycolic acid (PLGA) and Pluronic L121 (2%, v/v) as a surfactant by homogenization (Powergen 700, Fisher Scientific International Inc., Hampton, NH) for 60 s. The primary emulsion was added to 1% polyvinyl alcohol (PVA) solution saturated by DCM, and the solution was homogenized for 90 s to get a secondary emulsion. The secondary emulsion was rapidly stirred and the solvent was allowed to evaporate at room temperature (RT) for 3 h. IL-12-loaded microspheres were collected by filtration and washed with deionized water. The microspheres were then freeze-dried under vacuum (–49°C and <1 umHg) using a standard freeze-drier after freezing in liquid nitrogen or dried in a vacuum oven (Precision Scientific, Chicago, IL) at RT. Scanning electron micrographs demonstrated that the microspheres were 10～50 um in diameter. Recombinant mouse IL-12 protein and RIA-grade BSA were obtained from R&D Systems Inc. (Minneapolis, MN) and Sigma-Aldrich Co. (St. Louis, MO), respectively. PLGA having a 50/50 M composition of lactic/glycolic acid and MW 10,000 (RG502H) was purchased from Boehringer Ingelheim (Germany). Pluronic L121 was the product of

BASF (Parsippany, NJ). HPLC-grade DCM and methanol were obtained from Merck (Germany). Hydranal composite 5, PVA (88% hydrolyzed, MW 25,000) was the product of Sigma-Aldrich (St. Louis, MO).

ELISA. The levels of anti-S and anti-HA IgG antibodies (Abs) in the serum were monitored at different time points by ELISA as described (34,44). 0.1 ug/ml of HBsAg or 2 ug/ml of influenza HA in PBS was used as coating antigen. For semi-quantitative determination of anti-S and anti-HA antibody responses, endpoint titration of antibodies was performed with three-fold serial dilutions. Positive cutoff was set to have a higher absorbance than that of preimmune sera plus three standard deviations. The levels of IL-12p70 in the serum were measured using 'Quantikine Immunoassay kits' (M1270, R&D systems).

Lymphocytes isolation. Spleens were harvested from mice at various times after immunization or challenge, mechanically disrupted, passed through a 100-μm pore size cell strainer, and treated with buffered ammonium chloride solution to lyse the erythrocytes. Lung lymphocytes were isolated as previously described (7).

Magnetic bead separation of $CD8^+$ T cells. $CD8^+$ T cells were purified from vaccinated mice by positive selection. In brief, splenocytes or lung lymphocytes were prepared and incubated with anti-CD8 magnetic beads (Miltenyi Biotec) at 50 ul/10^8 total cells in 500 ul of magnetic bead separation (MACs) buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA) for 15 min at 4°C. Cells were washed with MACs buffer, resuspended in 500 ul MACs buffer and loaded onto pre-equilibrated MS^+ MACs column placed in a MACs separation unit. After washing, the magnetically bound cells were eluted from the column with the supplied plunger.

ELISPOT assay. ELISPOT assay was performed as described before (34). Splenocytes or lung lymphocytes were applied to plates with stimulants. The number of responsive cells was calculated by subtracting the mean number of spots induced in the absence of the peptide pool from that in the presence of the stimulants. The IFN-γ ELISPOT responses to medium controls were consistently $<10\%$ of the responses to peptide stimulation (data not shown).

Flow cytometry and tetramer staining. RSV MHC class-I-peptide tetramers were produced as described previously (2). RSV M2/82-90-specific tetramer positive cells were determined as previously described (7). To determine the number of influenza-specific IFN-γ-producing cells by intracellular cytokine staining, 2×10^6 freshly explanted lung lymphocytes were stimulated with influenza HA peptide (IYSTVASSL) for 5 h at 37°C in the presence of Brefeldin A, washed, surfaced stained with anti-CD8 antibody at 4°C and fixed/permeabilized using Fix/perm solution (FACSLyze (BD) diluted to 2× concentration with DW and 0.05% Tween 20 (Sigma)). Cells were washed once and incubated at room temperature with directly conjugated antibodies specific for IFN-γ. Cells were washed and resuspended in PBS containing 1% paraformaldehyde. Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed using CellQuest (BD Biosciences) and WinMDI software. All fluorochrome-conjugated monoclonal Abs were purchased from BD PharMingen (San Diego, CA).

Virus challenge. Immunized mice were challenged with RSV or influenza viruses as described (7,29). In brief, M2/82-90 peptide-immunized mice were lightly anesthetized with 2 : 1 mixture of ether and chloroform, and intranasally infected with 1×10^6 pfu of RSV viruses. For measurement of mouse weights, mice were monitored daily. Influenza HA-immunized mice were intranasally infected with 100 LD_{50} of mouse-adapted PR/8/34 influenza virus which corresponded to approximately 1600 plaque forming units (pfu). These viruses were plaque purified and grown in a MDCK cell line in order to maintain maximum virulence. The infected mice were then monitored every day to detect pathological conditions and for vitality after challenge. Statistical analysis was performed by Fisher's exact test.

Results

Encapsulation of IL-12 into PLGA increases $CD8^+$ T cell responses in RSV peptide vaccination. To investigate the effect of IL-12EM as an adjuvant for subunit vaccine, rIL-12 protein was encapsulated into poly (DL-lactic-co-glycolic acid) (PLGA) as a biodegradable microsphere in order to achieve the sustained release of IL-12. Recombinant IL-12 was encapsulated at a ratio of 40 ng of IL-12 per 1 mg of microspheres. To determine the *in vivo* phamacokinetics of IL-12 from r-IL-12 and IL-12EM, BALB/c mice (n=2/each group) were injected intranasally, subcutaneously or intramuscularly with rIL-12 or IL-12EM containing the same amount of IL-12 (0.5 ug). At the indicated time points after

injection (6 h, 12 h, 1 d, 2 d, 3 d, 4 d, 6 d, 8 d and 10 d), IL-12 concentrations in sera were measured using α-IL-12p70 ELISA (Fig. 1). As expected, a peak level of IL-12 in the mice injected with rIL-12 was observed within 12 h, and then its level was rapidly declined. In contrast, the release of IL-12 from IL-12EM was relatively slow, but persisted over 10 days, indicating that controlled release of IL-12 could be achieved *in vivo* by using PLGA encapsulation (Fig. 1).

In order to compare the adjuvant effect of IL-12EM with rIL-12 upon the induction of virus-specific $CD8^+$ T cell activity, BALB/c mice (n=9/group) were immunized intranasally twice at intervals of two weeks with 20 ug of $H\text{-}2^d$ restricted CTL epitope of respiratory syncytial virus (RSV) M2 protein (M2/82-90, SYIGSI NNI) plus 0.1 ug of rIL-12 (group 3) or IL-12EM (containing 0.1 ug of rIL-12) (group 4). Groups injected with M2 peptide alone (group 1) or M2 peptide plus mock-microspheres (group 2) were included as control groups. At two weeks after boost immunization, the frequency of $CD8^+$ T cell gated RSV M2/82-90 specific-CD62L- T cells in lung lymphocytes were measured by MHC tetramer staining (Fig. 2A). Group 1 and 2 did not show any significant M2/82-90-specific $CD8^+$ T cell response, compared to naïve group. These results indicate that M2 peptide alone is not immunogenic in our experimental conditions and that mock-microsphere itself does not augment the peptide vaccine-induced immune responses. As expected, M2/82-90 peptide plus rIL-12 (group 3) showed a slightly enhanced M2/82-90-specific $CD8^+$ T cell response. However, these $CD8^+$ T cell responses were dramatically enhanced by encapsulation of IL-12 in to PLGA (group 4) (1.08% vs. 8.45%) (Fig. 2A).

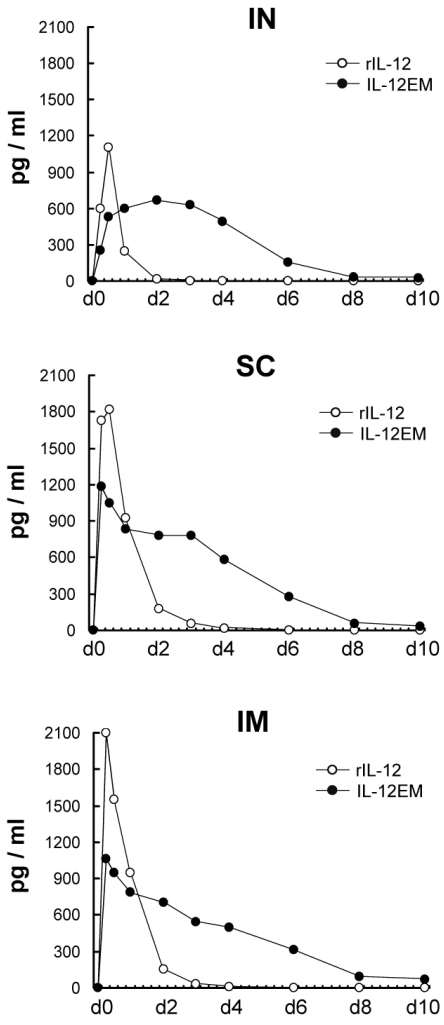

Figure 1. Encapsulation of rIL-12 into PLGA (IL-12EM) results in slow release of IL-12. BALB/c mice (n=2/each group) were injected intranasally (IN), subcutaneously (SC) or intramuscularly (IM) with rIL-12 or IL-12EM. At the indicated time points after injection, the level of IL-12 in sera was measured by α-IL-12p70 ELISA.

To address whether the enhanced $CD8^+$ T cell response by codelivery of rIL-12EM is correlated with better protection against virus challenge, the immunized mice (n=5/group) were challenged with 1×10^6 PFU of RSV at 4 weeks after boost immunization. When weight loss as a disease score was measured daily followed by the RSV challenge, mice immunized with M2/82-90 peptide plus IL-12EM (group 4) showed significantly reduced loss in body weight compared to other groups (Fig. 2B). Collectively, these results demonstrate that co-administration of rIL-12EM can significantly enhance the antigen-specific $CD8^+$ T cell response and thus protection against respiratory virus infection compared to codelivery of rIL-12 protein.

Codelivery of IL-12EM increases both antibody and $CD8^+$ T cell responses in HBsAg protein vaccination. To investigate the effect of IL-12EM in protein vaccine that has been known to rarely prime CTL response, BALB/c mice (n=10/group) were immunized subcutaneously with 0.5 ug of commercially available HBV S subunit vaccine (Hepavax, Greencross co., Republic of Korea) plus IL-12EM (containing 0.1 ug of rIL-12). To monitor the enhancement of antigen-specific antibody and CTL responses, the level of serum IgG, its subtypes, and HBV S CTL epitope-specific IFN-γ-secreting $CD8^+$ T cells were measured. Groups injected with HBsAg

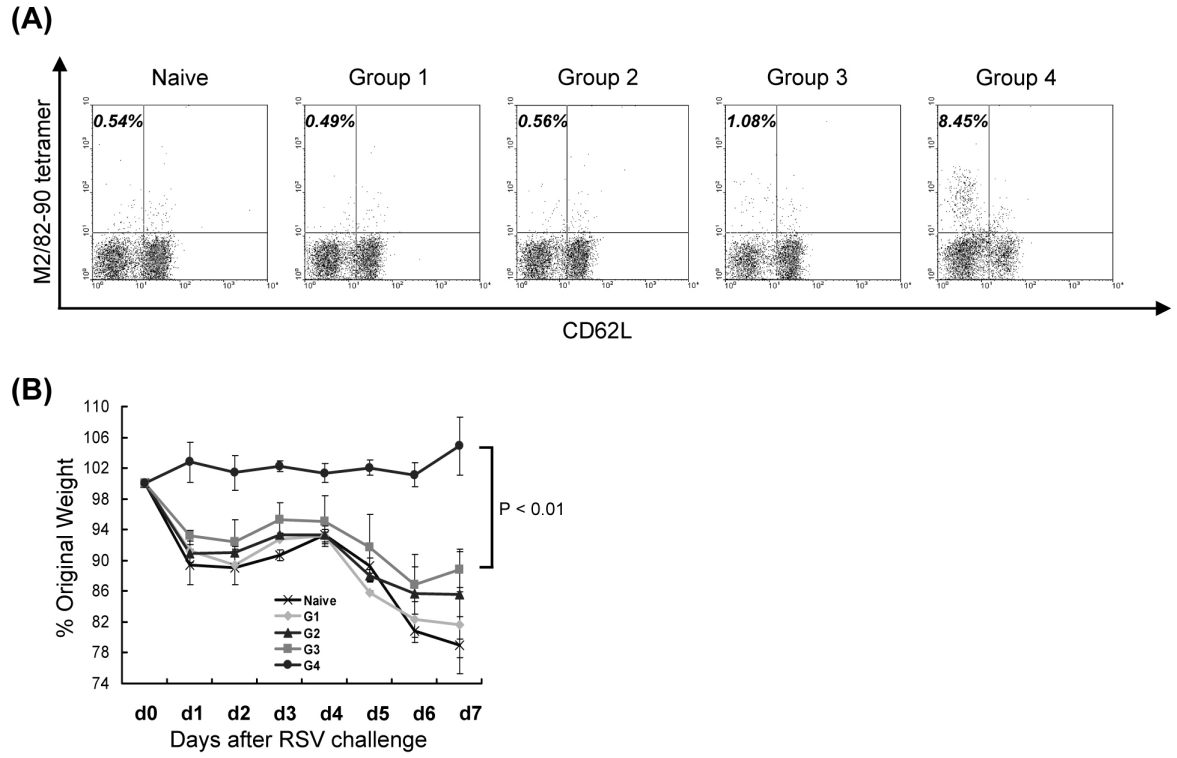

Figure 2. Encapsulation of rIL-12 into PLGA increases $CD8^+$ T cell responses and protection in RSV peptide vaccination. BALB/c mice (n=9/group) were immunized intranasally at intervals of two weeks as follows; Group 1: M2/82-90 peptide (20 ug), Group 2: M2/82-90 peptide (20 ug) + mock-microsphere, Group 3: M2/82-90 peptide (20 ug) + mock-microsphere + rIL-12 (0.1 ug), Group 4: M2/82-90 peptide (20 ug) + IL-12EM (0.1 ug). (A) At two weeks after boost immunization, lung lymphocytes were prepared, and the frequencies of $CD8^+$ T cell gated RSV M2/82-90 specific-CD62L- T cells were measured by MHC tetramer staining. (B) At 4 weeks after boosting, 5 mice of each group were infected with 1×10^6 pfu of RSV, and weight loss was monitored daily followed by RSV challenge. The percentage of weight loss was calculated based on the original weight of each mouse prior to challenge.

alone, HBsAg plus mock-microsphere, or HBsAg plus mock-microsphere plus rIL-12 were included as controls.

When we checked the levels of anti-S total IgG, IgG1, and IgG2a by ELISA using sera obtained at 5 weeks after immunization, mice injected with HBsAg alone showed significant anti-S total IgG and IgG1 responses, but not anti-S IgG2a responses, which may result from Th2-shifted immunity driven by alum in commercial HBV vaccine (Fig. 3A). As expected, mice injected with HBsAg plus mock microspheres (group 2) showed similar levels of antibody response compared with those injected with HBsAg alone, indicating that microspores alone have no immune-modulating effect. Codelivery of rIL-12 (group 3) showed slightly increased levels of anti-S total IgG, which is statistically insignificant ($p>0.1$). In terms of IgG2a response, 2 out of 10 mice in group 3 were seroconverted. Interestingly, the codelivery of IL-12EM (group 4) significantly enhanced total IgG, IgG1, and IgG2a responses specific to HBV S antigen compared to other groups (Fig. 3A & 3B). More specifically, mice injected with HBsAg plus IL-12EM showed 9 times higher anti-S IgG1 endpoint titer compared to other groups of mice. This is consistent with the previous results that codelivery of rIL-12 increases antigen-specific IgG1 antibody response and IgG2a response (6). In terms of the anti-S IgG2a response, group 4 showed a seroconversion rate of 90% (9/10), and its response was found to be 100~2000 times stronger than that observed for other groups (Fig. 3A & 3B). In addition, mice in group 4 showed the highest ratio of IgG2a to IgG1, suggesting the induction of strong Th1 immunity (Fig. 3B-3).

To investigate the adjuvant effect of IL-12EM on the induction of long-term $CD8^+$ T cell response, the IFN-γ ELISPOT assay for HBV S-specific CTL epitope (S28-39, H-2^d-restrcied CTL epitope against HBVS protein : IPQSLPSWWTSL) at 13 weeks after immunization was performed using purified $CD8^+$ T cells from the spleen (Fig. 3C). CD8 cells from naïve mice

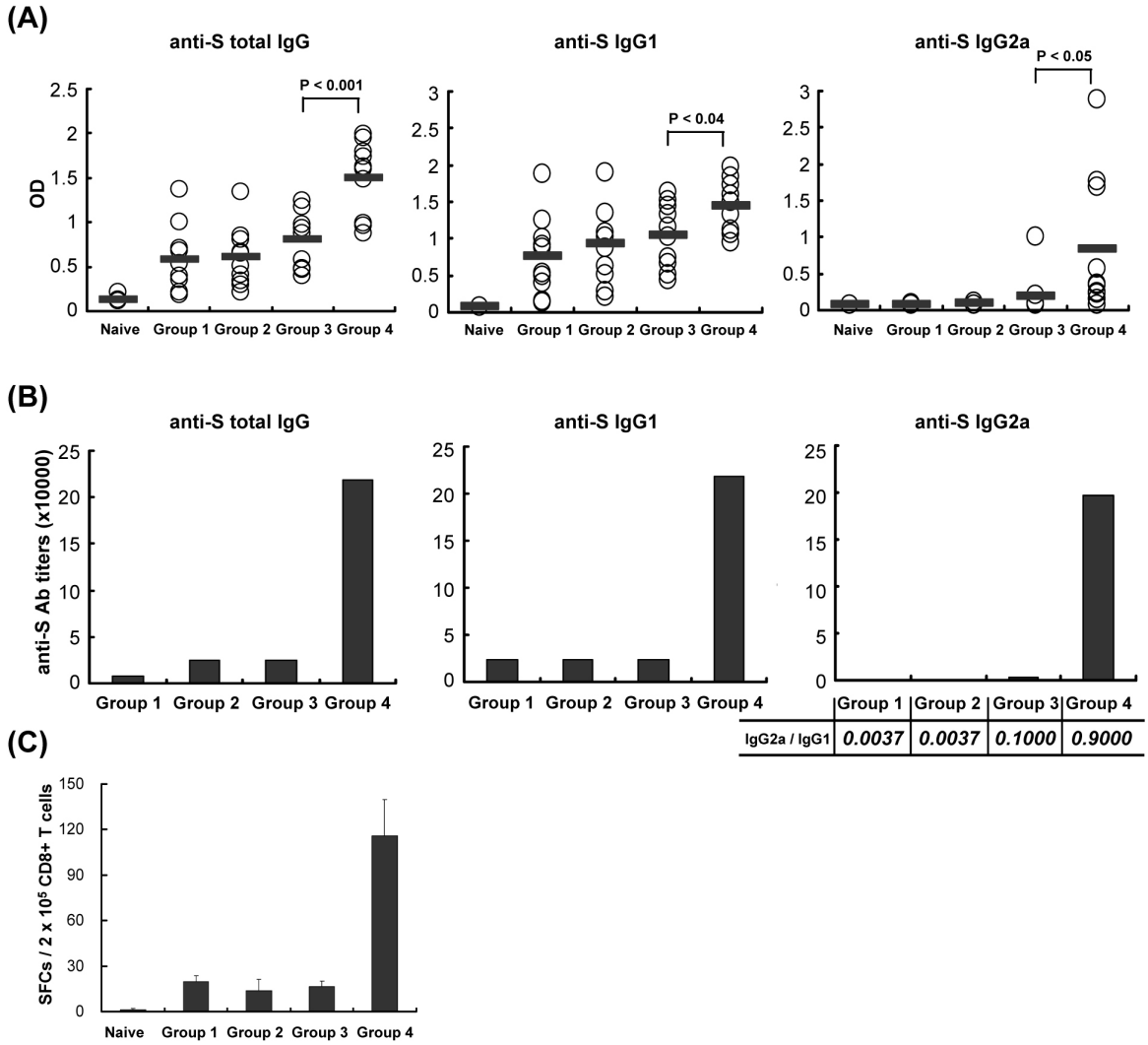

Figure 3. Codelivery of IL-12EM increases both antibody and $CD8^+$ T cell responses in HBsAg protein vaccination. BALB/c mice (n=10/group) were immunized subcutaneously as follows; Group 1: HBsAg (0.5 ug), Group 2: HBsAg (0.5 ug) + mock-microsphere, Group 3: HBsAg (0.5 ug) + mock-microsphere + rIL-12 (0.1 ug), Group 4: HBsAg (0.5 ug) + IL-12EM (0.1 ug). (A) At 5 weeks after immunization, anti-S total IgG, IgG1, and IgG2a antibody responses were measured at 5 weeks after immunization using ELISA. Ab responses were expressed as absorbance at 450 nm. (B) Anti-S total IgG, IgG1, and IgG2a end-point dilution titers using sera pool of each group were determined by end-point dilution assay. Ab responses were expressed as serum dilution factors. (C) To examine HBsAg-specific $CD8^+$ T-cell response, splenocytes were prepared at week 13, and $CD8^+$ T-cells were positively selected by magnetic bead separation. An IFN-γ ELISPOT assay was then performed using HBsAg-specific CTL epitope ($H\text{-}2^d$ restricted, S 28~39 a.a) as a stimulator. Results were expressed as the number of IFN-γ-secreting cells/2×10^5 $CD8^+$ T cells.

as a negative control exhibited no significant IFN-γ production in the presence of the peptide. Immunization with HBsAg alone was capable of inducing some $CD8^+$ T cell responses, which is consistent with the findings of a previous report (42). Codelivery of rIL-12 or mock microspheres did not have any effect on HBsAg-specific $CD8^+$ T cell response. However, codelivery of IL-12EM resulted in an exceedingly increased HBV S-specific $CD8^+$ T cell response. These enhanced HBsAg-specific $CD8^+$ T cell responses were observed until week 24 post-vaccination (data not shown); these findings are in good agreement with the previous report that the codelivery of IL-12 DNA elicited sustained memory CD8 T cell responses (16). Thus, our results indicate that the codelivery of IL-12EM was effective in the enhancement of both antibody and $CD8^+$ T cell responses in protein vaccination, such as HBsAg.

Codelivery of IL-12EM increases protection against virus challenge in influenza HA protein vaccination. To inves-

tigate whether codelivery of IL-12EM could result in enhanced immune responses in other types of protein vaccine such as influenza HA protein and increased protection against virus challenge, BALB/c mice (n= 10～13/group) were immunized intramuscularly two times, at intervals of two weeks with 9 ug of commercially available influenza HA subunit vaccine (LG Lifescience, Republic of Korea) plus IL-12EM (containing 0.1 ug of rIL-12) (group 3). Groups injected with HA vaccine alone (group 1) and HA plus mock microspheres plus 0.1 ug of rIL-12 (group 2) were included as control groups. As expected, group 2 mice showed slightly higher anti-HA antibody titers than group 1 mice. In addition, group 3 mice showed the highest anti-HA antibody titers and ratio of IgG2 to IgG1, which is similar with HBsAg vaccine (Fig. 4A).

To investigate whether the immune responses enhanced by codelivery of IL-12EM can lead to increased protection against influenza infection, the immunized mice were challenged with lethal doses of semi-homol-

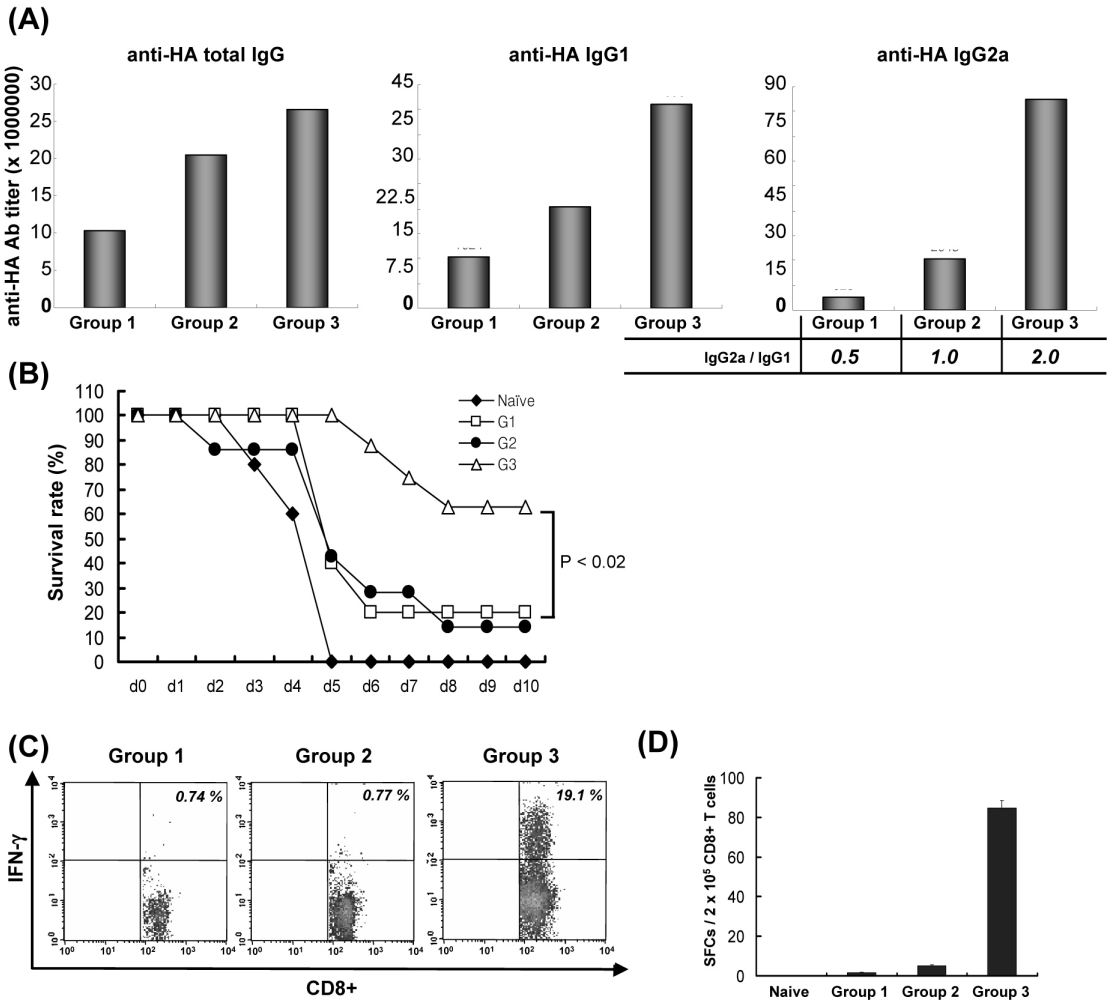

Figure 4. Codelivery of IL-12EM increases protection against virus challenge in influenza HA protein vaccination. BALB/c mice (n=10～13/group) were immunized intramuscularly two times at intervals of two weeks as follows; Group 1: HA (9 ug), Group 2: HA (9 ug) + mock-microsphere + rIL-12 (0.1 ug), Group 3: HA (9 ug) + IL-12EM (0.1 ug). (A) At 10 weeks after boost immunization, anti-HA total IgG, IgG1, and IgG2a end-point dilution titers using sera pool of each group were determined by end-point dilution assay. Ab responses were expressed as serum dilution factors. (B) At 11 weeks after boost immunization, survival rates were monitored daily, followed by challenge with lethal doses of influenza virus. (C) At 3 days after influenza challenge, lung lymphocytes were prepared, and the frequency of HA specific-IFN-γ-secreting $CD8^+$ T cells was measured by intracellular cytokine staining. (D) To examine HA-specific $CD8^+$ T cell responses in splenocytes, splenocytes were prepared at 3 days after influenza challenge, and $CD8^+$ T cells were positively selected by magnetic bead separation. The IFN-γ ELISPOT assay was then performed using HA-specific CTL epitope ($H\text{-}2^d$ restricted) as a stimulator. Results were expressed as the number of IFN-γ-secreting cells/2×10^5 $CD8^+$ T cells.

ogous influenza virus (H1N1, A/PR8/34 strain) at 11 weeks after boost immunization (Fig. 4B). As a negative control, naïve mice showed a survival rate of 0% (0/5) within 5 days after influenza challenge. Group 1 and group 2 mice showed slightly enhanced survival rates compared with the naive group (20%, 2/10 and 14%, 1/7, respectively). As expected, group 3, which was injected with HA plus IL-12EM, showed a significantly enhanced survival rate (63%, 5/8), and this difference was statistically significant (group 1 vs. group 3, $p < 0.02$). To examine the correlation between survival rate after virus challenge and vaccine-induced T cell immunity, splenocytes and lung lymphocytes were prepared from the challenged mice of each group (n=3/group) at 3 days after virus challenge. HA-specific $CD8^+$ T cell responses were measured by IFN-γ intracellular staining and ELISPOT assay stimulated with HA-specific CTL epitope (HA533-541, H-2^d-restricted CTL epitope against influenza HA protein : IYSTVASSL) (Fig. 4C & 4D). Interestingly, group 3 showed a stronger HA-specific peripheral $CD8^+$ T cell response in the lung, the primary influenza infection site, compared to group 1 and group 2 (19.1% vs. 0.74%/0.77%) (Fig. 4C). Similar to the results obtained in lung lymphocytes, the number of IFN-γ-producing $CD8^+$ T cells specific to HA CTL epitope in splenocytes were increased approximately 14~50-fold compared to group 1 and group 2 (Fig. 4D), implying that the enhanced HA-specific $CD8^+$ T cell response by codelivery of IL-12EM might promote viral clearance. *Enhancement of $CD8^+$ T cell responses by IL-12EM is dependent on $CD4^+$ T cell "help" in protein vaccination*. To examine the underlying mechanism related to the enhancement of Ag-specific $CD8^+$ T cell responses by codelivery of IL-12EM in protein vaccination, C57BL/6 wildtype (group 1 and group 2) and $CD4^+$ T cell knockout mice (group 3 and group 4) were immunized intramuscularly two times at intervals of two weeks as follows; Group 1: HA (9 ug), Group 2: HA (9 ug) + IL-12EM (0.1 ug), Group 3: HA (9 ug), Group 4: HA (9 ug) + IL-12EM (0.1 ug). As expected, when HA-specific $CD4^+$ and $CD8^+$ T cell responses were measured by IFN-γ ELISPOT assay, group 2 mice showed significantly enhanced HA-specific $CD4^+$ and $CD8^+$ T cell responses compared to group 1 mice (Fig. 5A). However, these enhancements were completely absent in $CD4^+$ T cell knockout mice (group 3 vs. group 4) (Fig. 5A). These results indicate that the enhancement of the $CD8^+$ T cell response by IL-12EM in protein vaccines appears to depend on the $CD4^+$ T cells "help".

In terms of anti-HA antibody responses, the codelivery of IL-12EM (group 2) significantly enhanced total IgG responses specific to HA antigen in C57BL/6 mice (Fig. 5B). In addition, this effect was also observed in $CD4^+$ T cell knockout mice (group 4), although the increased response resulting from codelivery of IL-12EM was lower in $CD4^+$ T cell knockout mice than in C57BL/6 mice. This result indicates that enhancement of Ab responses by IL-12EM is partially

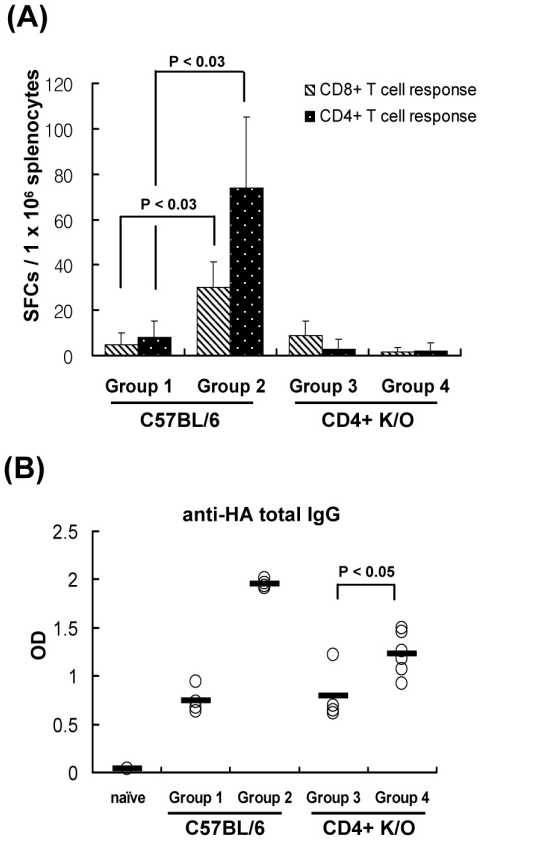

Figure 5. Enhancement of $CD8^+$ T cell responses by IL-12EM is dependent on $CD4^+$ T cell "help" in protein vaccination. C57BL/6 wildtype (group 1 and group 2) and $CD4^+$ T cell knockout mice (group 3 and group 4) were immunized intramuscularly two times at intervals of two weeks as follows; Group 1: HA (9 ug), Group 2: HA (9 ug) + IL-12EM (0.1 ug), Group 3: HA (9 ug), Group 4: HA (9 ug) + IL-12EM (0.1 ug). (A) At 3 weeks after boost immunization, $CD4^+$ and $CD8^+$ T cell responses were measured by IFN-γ ELISPOT assay stimulated with recombinant HA protein and CTL epitopes, respectively. (B) At 3 weeks after boost immunization, anti-HA total IgG antibody responses were determined using ELISA. Ab responses were expressed as absorbance at 450 nm.

independent on $CD4^+$ T cell "help" than that of $CD8^+$ T cell responses.

Discussion

Our data indicate that the biodegradable IL-12EM in peptide and protein vaccines can dramatically enhance antigen-specific $CD8^+$ T cell as well as antibody responses. Although the importance of IL-12 in the control of infection has been widely accepted, at present, recombinant IL-12 has not been regarded as an effective vaccine adjuvant because of its transient effect. It is worth noting that this limitation of recombinant IL-12 as a vaccine adjuvant can be overcome by the use of IL-12EM. A precondition for the successful implementation of subunit vaccine may lie in the generation of long-term immunological memory response. This point has been a particular cause of concern in the development of an effective protein vaccine, since protein vaccines could induce high levels of immunity immediately after vaccination; followed by rapid diminishment of vaccine-induced immunity over time. It is worth noting that significant levels of HBsAg-specific $CD8^+$ T cell responses were maintained up to 24 weeks after immunization by codelivery of IL-12EM in mice injected with HBsAg (data not shown), which might be caused by to the sustained existence of IL-12 from microspheres. It is supported by a previous report that long-term expression of IL-12 was able to maintain the memory T cell response for a longer period of time (15).

Our results suggested that enhanced $CD4^+$ T cell responses by codelivery of IL-12EM play an essential role in the induction of strong and sustained $CD8^+$ T cell responses in protein vaccination. It was reported that tumor, viral, and minor transplantation antigens are known to enter the class I-restricted antigen presentation pathway through "cross-presentation" under certain circumstances (25). While the priming of CTL to certain viruses or antigens via cross-presentation occurred in the absence of $CD4^+$ T cell "help" (19,39), the majority of CTL priming by cross-presentation appears to be facilitated by the participation of $CD4^+$ T cell "help" (13,14,40), which agrees well with our results that enhanced $CD8^+$ T cell responses to protein antigen by IL-12EM completely disappeared in $CD4^+$ T cell knockout mice. In order for APCs to cross-prime exogenous antigens, APCs were need to be activated by stimuli such as immune complex (11), $CD4^+$ T cell help via CD40- CD40L interaction (37), TLR ligands (10) or other undefined signals (33). Among them, "licensing" of APCs by Th1 $CD4^+$ T cells leads to the release of exogenous antigens from endocytic vesicles into their cytosol for efficient class I cross-presentation (4,32,38). Recently, we demonstrated that protein vaccine-induced Th1 $CD4^+$ T cell response was dramatically increased by codelivery of IL-12EM (17), which is consistent with a previous report that sustained IL-12 signaling is required for Th1 development (3). IL-12 was well known to promote IFN-γ secretion from $CD4^+$ T cells which, in turn, potently stimulate APC for further production of IL-12 by positive feedback loop, resulting further increased generation of Th1 $CD4^+$ T cells (24,31). Thus, it is likely that sustained IL-12 signaling by IL-12EM might contribute to altering the function of APC for cross-priming to exogenous antigens via enhanced Th1 $CD4^+$ T cell responses. Regarding the enhanced antibody responses by IL-12EM, it was reported that IL-12 directly affects humoral immunity by the binding of IL-12 to IL-12 receptor on B lymphocytes (41), suggesting the possible underlying mechanism about the partial dependence of $CD4^+$ T cell responses (Fig. 5B). However, in case of RSV peptide vaccine model, action mechanisms related to the increased RSV M2-specific CTL responses by codelivery of IL-12EM might be different from protein vaccine, since $CD8^+$ CTL induction by peptide vaccine does not require "cross-priming" pathway. As, it has been suggested that appropriate inflammatory and/or other danger signals are necessary to stimulate naive CTL precursors in response to CTL epitope peptide (5, 23,26) and IL-12 has been suggested as a "danger signal" for the activation of naive $CD8^+$ T cells (9), it is likely that sustained release of rIL-12 may result in the activation of naive $CD8^+$ T cells to induce CTL responses to peptide antigen.

It was reported that low doses of native HBsAg lipoprotein particles without adjuvants applied to $H\text{-}2^d$ mice could prime CTL by "cross-priming" in a $CD4^+$ T cell-dependent manner (42). Since commercially available HBV vaccine used in this experiment (Hepavax, Greencross Co., Republic of Korea) contains alum hydroxide, the $CD4^+$ T cell response might be shifted to the Th2 typed-immune response, resulting in inefficient priming of CTLs against HBsAg. In this re-

gard, it is interesting to note that $CD8^+$ T cell responses induced by HBsAg plus alum hydroxide are not as high as those seen in the previous report (42). Considering that IL-12 is known to be a strong Th1 vaccine adjuvant, Th2-shifted immunity driven by alum could be circumvented by *in vivo* persistence of IL-12. Thus, combination of IL-12EM and alum hydroxide might be a promising strategy for efficient enhancement of the $CD8^+$ T cell response, as well as the antibody response in the protein vaccine model.

Another novel approach for achieving *in vivo* persistence of rIL-12 is IL-12 DNA vaccination. It has been reported that protein and peptide vaccines induced efficient CTL activity by codelivery of IL-12 DNA vaccine compared to IL-12 protein (15,18). To compare the relative adjuvant effect between IL-12EM and IL-12 DNA, the anti-S antibody responses (total IgG, IgG1, and IgG2a) were investigated after vaccination with HBsAg plus IL-12EM or IL-12 DNA vaccine. Codelivery of IL-12 DNA or IL-12EM significantly enhanced anti-S total IgG, IgG1, and IgG2a antibody responses compared to HBsAg alone (data not shown). However, the adjuvant effect of IL-12 DNA appeared to be significantly lower than that of IL-12EM, presumably due to the low level of *in vivo* IL-12 expressed by IL-12 DNA (data not shown).

In conclusion, we showed that codelivery of IL-12EM enhanced both the antibody and $CD8^+$ T cell responses that lead to protection against challenge with viruses such as RSV and influenza virus. This is the first report to demonstrate that IL-12EM as an adjuvant of protein or peptide vaccine is effective in inducing a strong antigen-specific $CD8^+$ T cell response as well as protection against virus infection. Thus, IL-12EM as an novel vaccine adjuvant is worth further clinical investigation wherein dramatically increased long-lasting CTL as well as antibody responses are predicted to improve preventive efficacy of current protein-based vaccine. Furthermore, the same approach may be applicable to cancer or other infectious viral diseases such as HCV and HIV. Thus, this promising results may lay the groundwork for introducing more effective subunit vaccines in the near future.

Acknowledgments

We thank Sang Chun Lee and Kwan Seok Lee for the dedicated animal care and Sang Jun Ha for the helpful discussion. We thank Sol Kim for the technical support.

References

1. Afonso LC, Scharton TM, Vieira LQ, Wysocka M, Trinchieri G, Scott P: The adjuvant effect of interleukin-12 in a vaccine against Leishmania major. Science 263;235-237, 1994
2. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM: Phenotypic analysis of antigen-specific T lymphocytes. Science 274;94-96, 1996
3. Athie-Morales V, Smits HH, Cantrell DA, Hilkens CM: Sustained IL-12 signaling is required for Th1 development. J Immunol 172;61-69, 2004
4. Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR: Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med 186;65-70, 1997
5. Berzofsky JA, Ahlers JD, Belyakov IM: Strategies for designing and optimizing new generation vaccines. Nat Rev Immunol 1;209-219, 2001
6. Bliss J, Van Cleave V, Murray K, Wiencis A, Ketchum M, Maylor R, Haire T, Resmini C, Abbas AK, Wolf SF: IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. J Immunol 156;887-894, 1996
7. Chang J, Choi SY, Jin HT, Sung YC, Braciale TJ: Improved effector activity and memory CD8 T cell development by IL-2 expression during experimental respiratory syncytial virus infection. J Immunol 172;503-508, 2004
8. Crotts G, Park TG: Protein delivery from poly (lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues. J Microencapsul 15;699-713, 1998
9. Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK, Mescher MF: Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J Immunol 162;3256-3262, 1999
10. Datta SK, Redecke V, Prilliman KR, Takabayashi K, Corr M, Tallant T, DiDonato J, Dziarski R, Akira S, Schoenberger SP, Raz E: A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. J Immunol 170;4102-4110, 2003
11. den Haan JM, Bevan MJ: Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(−) dendritic cells in vivo. J Exp Med 196;817-827, 2002
12. Egilmez NK, Jong YS, Sabel MS, Jacob JS, Mathiowitz E, Bankert RB: In situ tumor vaccination with interleukin-12-encapsulated biodegradable microspheres: induction of tumor regression and potent antitumor immunity. Cancer Res 60; 3832-3837, 2000
13. Fayolle C, Deriaud E, Leclerc C: In vivo induction of cytotoxic T cell response by a free synthetic peptide requires CD4+ T cell help. J Immunol 147;4069-4073, 1991
14. Guerder S, Matzinger P: A fail-safe mechanism for maintaining self-tolerance. J Exp Med 176;553-564, 1992
15. Gurunathan S, Prussin C, Sacks DL, Seder RA: Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. Nat Med 4;1409-1415, 1998
16. Ha SJ, Chang J, Song MK, Suh YS, Jin HT, Lee CH, Nam GH, Choi G, Choi KY, Lee SH, Kim WB, Sung YC: Engineering N-glycosylation mutations in IL-12 enhances sustained cytotoxic T lymphocyte responses for DNA immunization. Nat Biotechnol 20;381-386, 2002

17. Ha SJ, Park SH, Kim HJ, Kim SC, Kang HJ, Lee EG, Kwon SG, Kim BM, Lee SH, Kim WB, Sung YC, Cho SN: Enhanced immunogenicity and protective efficacy with the use of interleukin-12-encapsulated microspheres plus AS01B in tuberculosis subunit vaccination. Infect Immun 74;4954-4959, 2006
18. Hamajima K, Fukushima J, Bukawa H, Kaneko T, Tsuji T, Asakura Y, Sasaki S, Xin KQ, Okuda K: Strong augment effect of IL-12 expression plasmid on the induction of HIV-specific cytotoxic T lymphocyte activity by a peptide vaccine candidate. Clin Immunol Immunopathol 83;179-184, 1997
19. Haskova Z, Usiu N, Pepose JS, Ferguson TA, Stuart PM: CD4+ T cells are critical for corneal, but not skin, allograft rejection. Transplantation 69;483-487, 2000
20. Heinzel FP, Schoenhaut DS, Rerko RM, Rosser LE, Gately MK: Recombinant interleukin 12 cures mice infected with Leishmania major. J Exp Med 177;1505-1509, 1993
21. Hill HC, Conway TF Jr, Sabel MS, Jong YS, Mathiowitz E, Bankert RB, Egilmez NK: Cancer immunotherapy with interleukin 12 and granulocyte-macrophage colony-stimulating factor-encapsulated microspheres: coinduction of innate and adaptive antitumor immunity and cure of disseminated disease. Cancer Res 62;7254-7263, 2002
22. Kim TH, Park TG: Critical effect of freezing/freeze-drying on sustained release of FITC-dextran encapsulated within PLGA microspheres. Int J Pharm 271;207-214, 2004
23. Knutson KL, Schiffman K, Disis ML: Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. J Clin Invest 107;477-484, 2001
24. Kubin M, Chow JM, Trinchieri G: Differential regulation of interleukin-12 (IL-12), tumor necrosis factor alpha, IL-1 beta production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood 83;1847-1855, 1994
25. Kurts C, Carbone FR, Barnden M, Blanas E, Allison J, Heath WR, Miller JF: CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. J Exp Med 186;2057-2062, 1997
26. Kyburz D, Aichele P, Speiser DE, Hengartner H, Zinkernagel RM, Pircher H: T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. Eur J Immunol 23;1956-1962, 1993
27. Langer R: Biomaterials in drug delivery and tissue engineering: one laboratory's experience. Acc Chem Res 33;94-101, 2000
28. Langer R: Drug delivery and targeting. Nature 392;5-10, 1998
29. Lee SW, Youn JW, Seong BL, Sung YC: IL-6 induces long-term protective immunity against a lethal challenge of influenza virus. Vaccine 17;490-496, 1999
30. Lindblad EB, Elhay MJ, Silva R, Appelberg R, Andersen P: Adjuvant modulation of immune responses to tuberculosis subunit vaccines. Infect Immun 65;623-629, 1997
31. Ma X, Chow JM, Gri G, Carra G, Gerosa F, Wolf SF, Dzialo R, Trinchieri G: The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. J Exp Med 183;147-157, 1996
32. Machy P, Serre K, Baillet M, Leserman L: Induction of MHC class I presentation of exogenous antigen by dendritic cells is controlled by CD4+ T cells engaging class II molecules in cholesterol-rich domains. J Immunol 168;1172-1180, 2002
33. Machy P, Serre K, Leserman L: Class I-restricted presentation of exogenous antigen acquired by Fcgamma receptor-mediated endocytosis is regulated in dendritic cells. Eur J Immunol 30;848-857, 2000
34. Park SH, Yang SH, Lee CG, Youn JW, Chang J, Sung YC: Efficient induction of T helper 1 CD4+ T-cell responses to hepatitis C virus core and E2 by a DNA prime-adenovirus boost. Vaccine 21;4555-4564, 2003
35. Partidos CD, Vohra P, Jones D, Farrar G, Steward MW: CTL responses induced by a single immunization with peptide encapsulated in biodegradable microparticles. J Immunol Methods 206;143-151, 1997
36. Putney SD, Burke PA: Improving protein therapeutics with sustained-release formulations. Nat Biotechnol 16;153-157, 1998
37. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ: T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 393;480-483, 1998
38. Serre K, Giraudo L, Siret C, Leserman L, Machy P: CD4 T cell help is required for primary CD8 T cell responses to vesicular antigen delivered to dendritic cells in vivo. Eur J Immunol 36;1386-1397, 2006
39. Song K, Chang Y, Prud'homme GJ: IL-12 plasmid-enhanced DNA vaccination against carcinoembryonic antigen (CEA) studied in immune-gene knockout mice. Gene Ther 7;1527-1535, 2000
40. Stuhler G, Schlossman SF: Antigen organization regulates cluster formation and induction of cytotoxic T lymphocytes by helper T cell subsets. Proc Natl Acad Sci U S A 94;622-627, 1997
41. Vogel LA, Showe LC, Lester TL, McNutt RM, Van Cleave VH, Metzger DW: Direct binding of IL-12 to human and murine B lymphocytes. Int Immunol 8;1955-1962, 1996
42. Wild J, Grusby MJ, Schirmbeck R, Reimann J: Priming MHC-I-restricted cytotoxic T lymphocyte responses to exogenous hepatitis B surface antigen is CD4+ T cell dependent. J Immunol 163;1880-1887, 1999
43. Yap G, Pesin M, Sher A: Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii. J Immunol 165;628-631, 2000
44. Youn JW, Park SH, Lavillette D, Cosset FL, Yang SH, Lee CG, Jin HT, Kim CM, Shata MT, Lee DH, Pfahler W, Prince AM, Sung YC: Sustained E2 antibody response correlates with reduced peak viremia after hepatitis C virus infection in the chimpanzee. Hepatology 42;1429-1436, 2005