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# Maturation and migration of dendritic cells upon stimulation with heat-killed tumor cells

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Recently it has been reported that immunization with heat-killed tumor cells (HK vaccine) induces anti-tumor immune responses in mice. To investigate how HK vaccine elicits anti-tumor specific adaptive immunity, we examined the effect of HK vaccination on innate immune cells such as dendritic cells (DCs), which are essential for the generation of adaptive immunity. Upon stimulation with HK vaccine, DCs matured to promote not only the up-regulation of co-stimulatory molecules but also secretion of cytokine IL12. Furthermore, HK vaccine-treated DCs migrated more efficiently to draining lymph nodes compared with untreated ones. Taken together, HK vaccine can be useful as an adjuvant to activate DCs for anti-tumor immune responses.

**Keywords:** heat-killed tumor cells; dendritic cells; interleukin 12

## Introduction

Dendritic cells (DCs) are the most potent APCs that direct the cellular immune response through antigen presentation in the presence of appropriate co-stimulation (Banchereau and Steinman 1998; Wan and Dupasquier 2005). DCs are activated by inflammatory stimuli or danger signals transmitted via innate immune receptors such as Toll-like receptors (TLRs) (Iwasaki and Medzhitov 2004; Jin and Lee 2009). After activation, DCs secrete cytokines such as IL12 via the NF-κB- and IFNγ-dependent pathways and migrate to T cell enriched areas of the secondary lymphoid organs where they foster the egress of activated, antigen-specific T lymphocytes into the periphery as effectors (Banchereau and Steinman 1998). It was recently demonstrated that tumor antigen-pulsed DCs primed MHC class II- and class I-restricted antigen-specific T cells in vivo and stimulated the regression of established renal cell carcinoma and melanoma (Strome et al. 2002).

In addition, IL12 produced by macrophages as well as DCs is a key cytokine in cellular immune responses. IL12 is a heterodimeric cytokine composed of two disulfide-linked subunits designated as p35 and p40 (Ribas et al. 2003; Langrish et al. 2004). IL12 promotes IFNγ production by a variety of immune cells including NK cells, $CD8^+$ CTLs and $CD4^+$ T cells (Ribas et al. 2003; Langrish et al. 2004). This suggests that DCs may be important target cells for the development of a clinically applicable cancer vaccine.

Recently, it was found that administration of heat-killed tumor cells (hereafter referred to as HK vaccine) into mice induced anti-tumor immune responses in the absence of adjuvants (Yoon et al. 2008). However, the underlying mechanism by which the HK vaccine elicits strong anti-tumor immune responses has not yet been investigated. Therefore, we decided to investigate how HK vaccine can induce potent anti-tumor immune responses. We focused on the effect of the HK vaccine on innate immunity, which is thought to be a requirement for the generation of adaptive immunity. To test whether the HK vaccine influences the activation of DCs, which are the key component for linking innate immune responses to adaptive ones, we took advantage of the cytokine reporter mouse model that was recently developed to track IL12p40-expressing cells with fluorescent marker proteins in vivo (Im et al. 2005; Reinhardt et al. 2006) (hereafter the IL12p40 reporter model is referred to as yet40).

Here, we have demonstrated that immunization of mice with the HK vaccine stimulates DCs and macrophages to induce IL12p40 expression and promotes expression of MHC and co-stimulatory molecules such as CD40 and CD86. Furthermore, we showed that HK vaccine may promote migration of DCs to secondary lymphoid organs to facilitate antigen presentation to antigen-specific T cells by modulating the expression levels of chemokine receptors such as CCR5 and CCR7. Collectively, these findings provide significant insight into the mechanism by which HK vaccine induces protective anti-tumor immune responses.



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## Materials and methods

### *Mice*

C57BL/6 (B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and Charles River Japan Inc. (Yokohama, Japan). IL12p40 reporter (yet40) mice were backcrossed over six generations to B6. B6 wild type and yet40 mice were bred in our animal facility and all the mice were 6–12 weeks of age at the time of experiments. The experiments involving animals were approved by the Institutional Animal Care and Use Committee at Sejong University (SJ-20100401009).

### *Reagents and cell lines*

Two TLR ligands were used as a positive control for in vitro stimulation assays in this study. CpG oligodeoxynucleotides (CpG) were manufactured by Geno Tech. (Daejun, Korea) and lipopolysaccharides (LPS) were purchased from Sigma (St. Louis, USA). CpG and LPS were used at a final concentration of 5 μg/ml and 1 μg/ml, respectively. B16-BL6 (B16) melanoma cells were purchased from American Type Culture Collection.

### *Preparation of HK vaccine*

HK vaccine was prepared as described elsewhere (Yoon et al. 2008). In brief, B16 cells were harvested and washed three times with PBS to remove serum contamination. The cells were then boiled in PBS for 30 minutes and washed several times with PBS to get rid of cell debris. After cell counting, heat-killed B16 cells were resuspended at $1 \times 10^7$ cells/ml in PBS for the experiment.

### *Generation of BMDCs*

Bone marrow-derived dendritic cells (BMDCs) were generated from the bone marrow cells of mice as described (Gilliet et al. 2002). Briefly, bone marrow cells were flushed with complete RPMI medium from femurs and tibiae of designated mice. After removal of red blood cells (RBCs) with an ACK lysis buffer (0.15 M $NH_4Cl$, 10 mM $KHCO_3$, 2 mM EDTA), bone marrow cells were washed with PBS and seeded at a concentration of $1 \times 10^6$ cells/ml in medium supplemented with recombinant mouse Flt3L (100 ng/ml; R&D Systems) or GM-CSF (40 ng/ml; R&D Systems) plus IL4 (10 ng/ml; R&D Systems) in a 24-well tissue culture plate. To generate BMDCs cultured with GM-CSF and IL4, half of the medium was removed and fresh cytokine-supplemented culture medium was added into the cultures at days 2 and 4. At day 5, the BMDCs were harvested and stimulated for 16 hrs with the indicated reagents in a U-bottom 96-well plate. To generate BMDCs cultured with Flt3L, fresh cytokine-supplemented culture medium was added at days 5 and 10. At day 12, the BMDCs were harvested, subsequently incubated with the indicated reagents, and analyzed at the specified time point by flow cytometry.

### *Preparation of splenic DCs*

Spleens were isolated from mice and subsequently single-cell suspensions of splenocytes were prepared using frosted microscope slides (Fisher, USA). After removal of RBCs with an ACK lysis buffer, the splenocytes were resuspended in RPMI complete medium. $CD11c^+$ splenic DCs were enriched using CD11c MACS beads (Miltenyi Biotec). Positively selected DCs were incubated at 37°C for 16 hrs at 1:2 ratio to HK vaccine in a U-bottom 96-well plate.

### *In vivo migration of DCs*

The Flt3L-cultured BMDCs were labeled with 5 μM 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions (Molecular Probes, USA) and incubated with the indicated reagents at 37°C for 16 hrs. After stimulation, the cells were washed extensively in PBS and subsequently injected into the footpads of B6 mice. To monitor the migration of the injected cells, draining popliteal lymph node cells were prepared 48 hrs post injection and then the total number of $CFSE^+$ cells was estimated by flow cytometry.

### *Flow cytometry*

Cells were stained with monoclonal antibodies (mAbs) for 1 hr on ice and subsequently washed three times with FACS wash buffer (PBS buffer containing 1% FBS). Cells were then fixed with 1% paraformaldehyde. The following mAbs were used in this study: phycoerythrin(PE)-conjugated anti-CD11c, PE-conjugated anti-CD8α, PE-conjugated anti-CD40, PE-conjugated anti-CD70, PE-conjugated anti-MHC I, PE-conjugated anti-MHC II, PE-conjugated anti-CCR5, PE-conjugated anti-CCR7, tricolor(TC)-conjugated anti-CD8α, TC-conjugated anti-CD11b, TC-conjugated streptavidin, TC-conjugated anti-B220, allophycocyanin(APC)–conjugated anti-CD11c, APC-conjugated anti-CD19 and biotinylated anti-CD80 and anti-CD86 mAbs. All flow cytometric data were acquired with a FACSCalibur flow cytometer (Becton Dickinson, USA) and analyzed with FlowJo software (Tree Star, USA). Anti-CD11c, anti-B220, anti-CD19, anti-CD11b, anti-CD8α, anti-CD40, and anti-CD70 mAb were purchased from BD Pharmingen (San Diego, CA, USA). Anti-CD80, anti-CD86,

anti-CCR5, anti-CCR7 were obtained from Caltag (Burlingame, CA, USA).

### *ELISA*

A standard sandwich enzyme-linked immunosorbent assay (ELISA) was used for measuring mouse IL12p40 levels in the serum of mice. Culture supernatants from cells stimulated with the indicated reagents for 16 hrs were measured for IL12p40 by ELISA (R&D Systems, USA).

## Results

### *HK vaccine induces IL12p40 gene expression in dendritic cells and peritoneal macrophages but not in B cells*

To elucidate the underlying mechanism by which the HK vaccine elicits anti-tumor immune responses, we first investigated whether the HK vaccine could activate APCs. APCs such as DCs play major roles in linking innate and adaptive immune responses (Banchereau and Steinman 1998; Liu 2001) and produce IL12 within hours after activation with TLR ligands such as LPS and CpG (Liu 2001; Langrish et al. 2004). We analyzed the expression of the IL12p40 gene in APCs to identify their activation levels in response to culture with the HK vaccine. For this purpose, we enriched $CD11c^+$ cells and $B220^+$ cells using magnetic beads from the spleens of reporter mice, cultured these cells for 16 hrs with HK vaccine, and then measured IL12p40 gene expression levels using flow cytometry. We found that the HK vaccine induced IL12p40 gene expression in DCs (Figure 1A) but not in B cells (Figure 1B). We also isolated peritoneal macrophages from reporter mice injected with 4% thioglycollate and incubated these cells overnight with HK vaccine. As with DCs, stimulation of macrophages with HK vaccine led to IL12p40 gene expression (Figure 1C). These data showed that the HK vaccine stimulated DCs and macrophages but not B cells to induce IL12p40 expression.

### *Preferential induction of IL12p40 gene expression by HK vaccine in lymphoid DCs rather than myeloid DCs*

In mice, splenic DC subsets can be classified into at least two subsets: myeloid related DCs ($CD11c^+CD11b^+CD8\alpha^-$) and lymphoid related DCs ($CD11c^+CD11b^-$ $CD8\alpha^+$). However, the absolute relationship between these two subsets remains unclear (Brasel et al. 2000; Shortman and Liu 2002; Kushwah and Hu 2011). Flt3L and GM-CSF as principal growth factors are used to generate DCs from bone marrow (BM) precursors in vitro (Banchereau and Steinman 1998; Brasel et al. 2000). These growth factors have opposing effects on the development of $CD11c^+CD11b^-$ lymphoid DCs and $CD11c^+CD11b^+$ myeloid DCs (Gilliet et al. 2002). Flt3L generates a large number of $CD11c^+CD11b^-$ lymphoid DCs with better than 40% purity from mouse bone marrow cultures (Brasel et al. 2000). By contrast, GM-CSF promotes generation of $CD11c^+CD11b^+$ myeloid DCs and completely blocks the development of lymphoid DCs (Brasel et al. 2000).

To investigate what types of DCs are activated by HK vaccine, we cultured BM cells derived from reporter mice in the presence of either Flt3L or GM-CSF. Flt3L or GM-CSF cultured BMDCs were then stimulated with CpG, HK vaccine, or medium only. Whereas HK vaccine stimulated Flt3L-cultured BMDCs to induce IL12p40 expression, HK vaccine was unable to induce IL12p40 expression in GM-CSF cultured BMDCs (Figure 2A). This finding was confirmed by measuring the amount of IL12p40 protein in the culture supernatants using ELISA. These results showed that HK vaccine induced increased levels of IL12p40 in Flt3L-cultured BMDCs more than in GM-CSF cultured BMDCs (Figure 2C). We speculate that the HK vaccine may be active only on lymphoid DCs because Flt3L generates both myeloid BMDCs and lymphoid BMDCs whereas GM-CSF produces only myeloid BMDCs. To test this possibility under more physiological conditions, we isolated primary $CD11c^+$ cells from the spleens of reporter mice using MACS. Purified $CD11c^+$ cells were stimulated with HK vaccine and 16 hrs later IL12p40 expression was evaluated on $CD11c^+CD11b^-$ or $CD11c^+CD11b^+$ gated DC populations. As expected, HK vaccine induced IL12p40 expression mainly in the $CD8\alpha^+CD11c^+CD11b^-$ population but not in the $CD8\alpha^-$ $CD11c^+CD11b^+$ population (Figure 2B). These results are consistent with our finding above, providing evidence that lymphoid DCs are the main producers of IL12 responsible for inducing Th1 immune responses. We also measured the amount of IL12p40 in the culture supernatants by ELISA. Induction of IL12p40 by HK vaccine was confirmed in splenic DCs (Figure 2C). Since $CD8\alpha^+$ DCs activated by the HK vaccine play a specialized role in stimulating cytotoxic T lymphocytes (Colonna et al. 2006), HK vaccine may induce CTL-mediated anti-tumor immune responses through the activation of $CD8\alpha^+$ DCs.

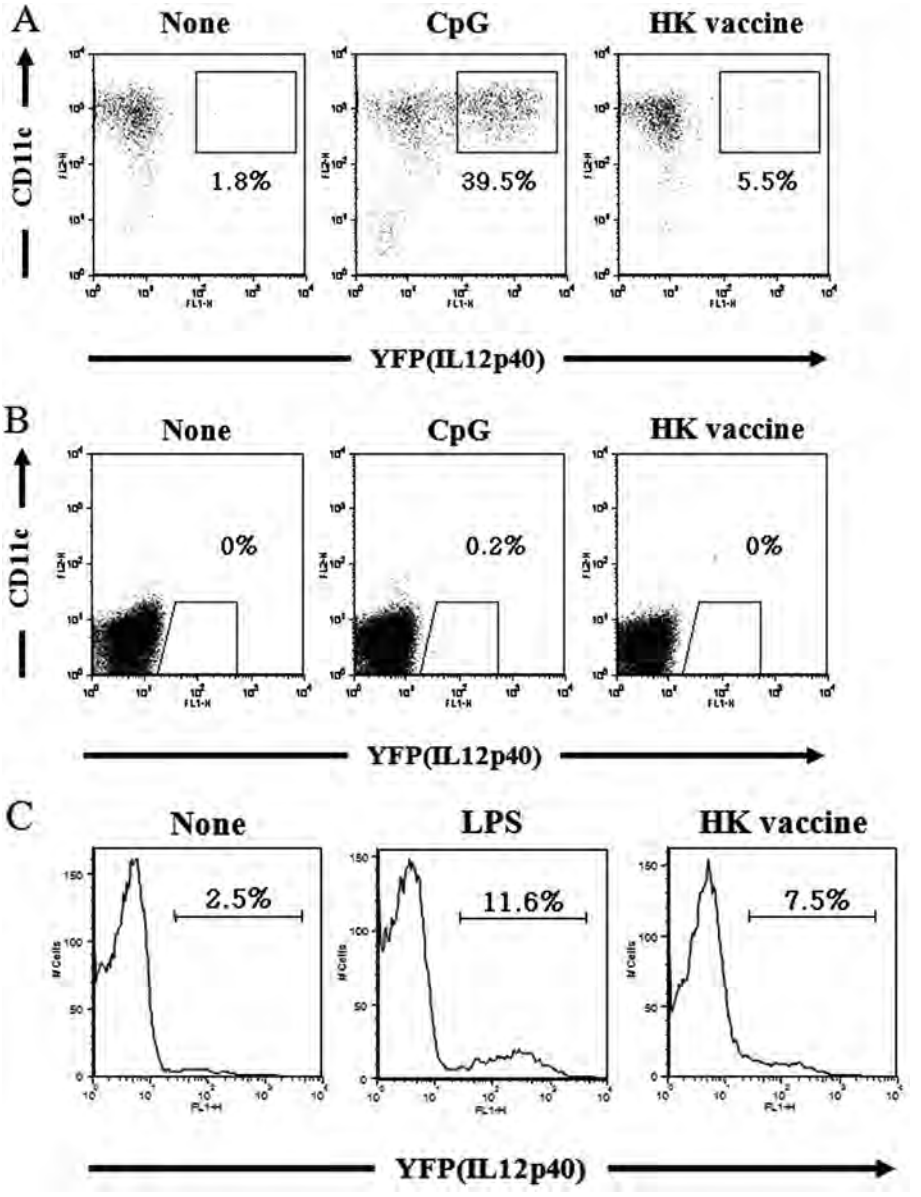


Figure 1. Induction of IL12p40 gene in DCs and macrophages by HK vaccines. $CD11c^+$ cells (A) and $B220^+$ cells (B) were enriched from the spleens of reporter mice by positive selection using MACS magnetic beads. Positively selected cells were stimulated with CpG (5 μg/ml), HK vaccines, or medium only for 16 hrs. To enrich peritoneal macrophages (C), reporter mice were injected i.p. with 4% thioglycollate. Three to 5 days later, peritoneal macrophages were isolated from the abdominal cavity. Isolated macrophages were stimulated with LPS (1 μg/ml), HK vaccines, or medium only for 16 hrs. IL12p40 expression was analyzed on $CD19^+B220^+$ (B) or $CD11b^+CD11c^-$(C) gated cells and evaluated by flow cytometry for YFP expression. Data represent one of three independent experiments.

### *HK vaccine induces maturation of both DCs and macrophages*

Full activation of T cells requires not only cognate interactions between a TCR and MHC-peptide complex, but also interactions between a co-stimulatory receptor on the surface of the T cell and a co-stimulatory ligand on APC (Hurwitz et al. 2000; Hoebe et al. 2004). Hence, the development of antigen-specific T cell responses requires maturation of DCs, which entails up-regulation of MHC class I and II molecules and co-stimulatory molecules such as CD80, CD86, CD40, and CD70 (Hoebe et al. 2004). To assess whether HK vaccine affects the maturation of DCs, we analyzed the expression levels of MHC and co-stimulatory molecules on Flt3L-cultured BMDCs and splenic DCs stimulated with CpG, HK vaccine or medium for 16 hrs. Both the Flt3L-cultured BMDCs

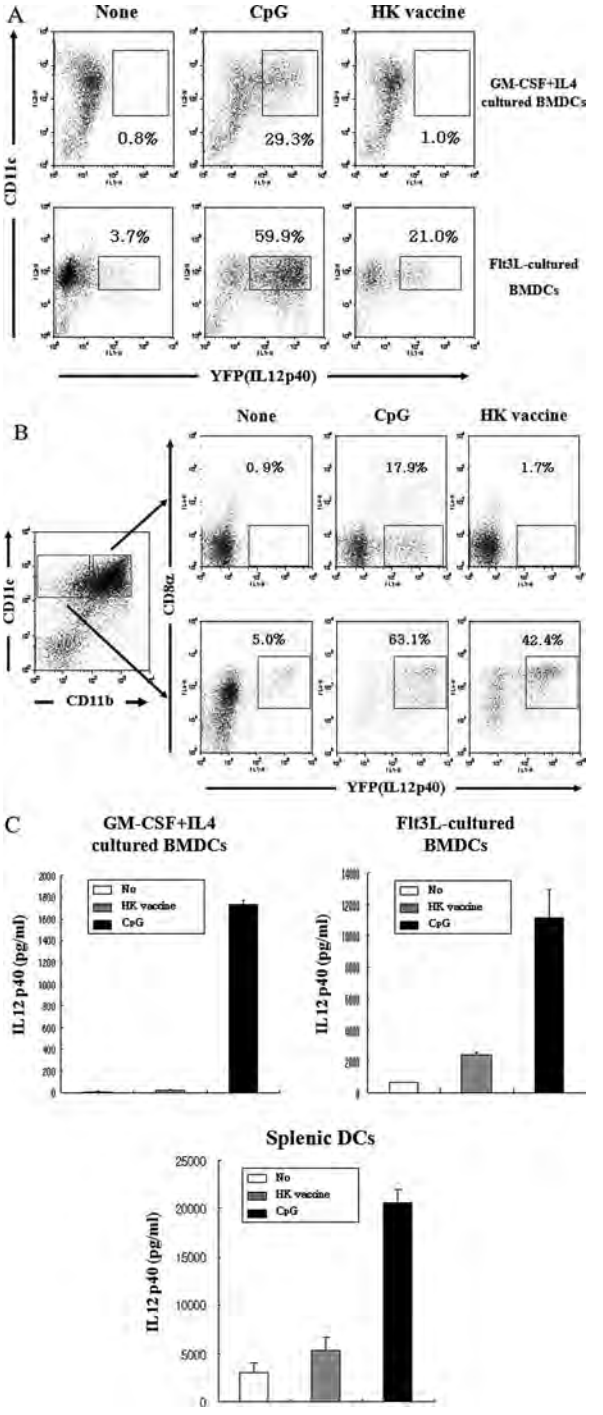


Figure 2. Preferential induction of IL12p40 gene expression on $CD8\alpha^+CD11c^+CD11b^-$ cells by HK vaccine. (A) To generate bone-marrow derived DCs, bone marrow (BM) cells were prepared from the tibia and femur of reporter mice. BM cells were subsequently cultured with Flt3L (100 ng/ml) or GM-CSF (40 ng/ml) plus IL4 (10 ng/ml). On day 12 or day 5 BMDCs were stimulated with CpG (5 μg/ml), HK vaccines, or medium only for 16 hrs and then stained with anti-CD11c mAbs. The frequency of IL12p40-expressing BMDCs was analyzed on $CD11c^+$ gated cells and determined by flow cytometry for YFP intensity. (B) $CD11c^+$ cells were enriched from the spleens of reporter mice using CD11c MACS magnetic beads and stimulated with CpG (5 μg/ml), HK vaccines, or medium only for 16 hrs. The percentage of IL12p40-expressing cells was examined in $CD11c^+CD11b^-$ or $CD11c^+CD11b^+$ DCs and determined by flow cytometry for YFP intensity. (C) Flt3L, GM-CSF plus IL4 cultured bone marrow-derived DCs (BMDCs) and splenic DCs cultured with CpG (5 μg/ml), HK vaccines, or medium only for 16 hrs were harvested, and production of IL12p40 proteins was determined by ELISA. Data shown represent the average $\pm$ SD ($n = 3$).

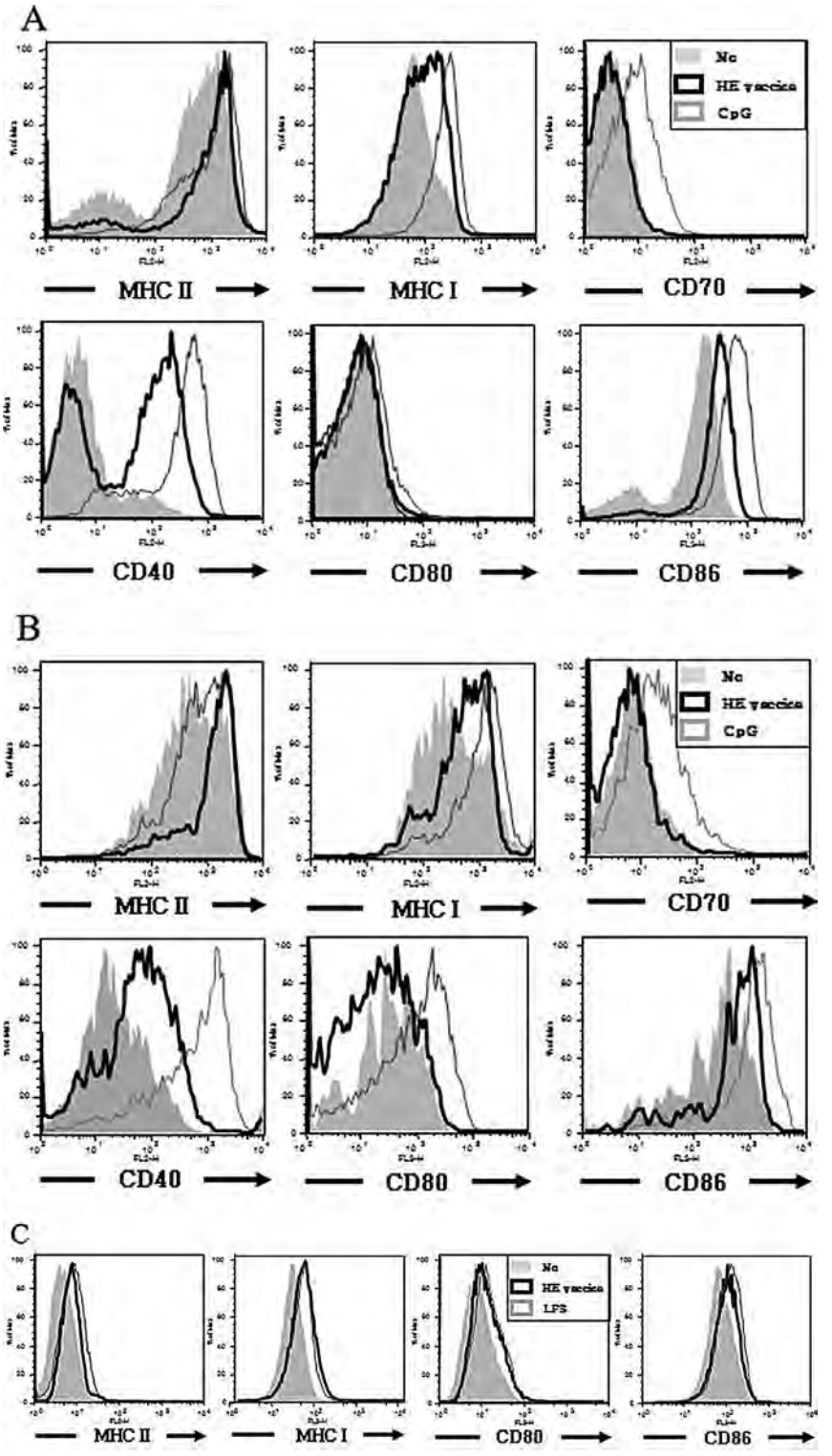


Figure 3. Maturation of DCs and macrophages by HK vaccine. (A) Flt3L-cultured BMDCs, (B) splenic DCs and (C) macrophage cell line J774 were cultured with CpG (5 μg/ml), HK vaccines, or medium only. Sixteen hours later, these cells were stained with anti-CD11c, anti-CD8α, anti-CD40, anti-CD70, anti-CD80, anti-CD86, anti-MHC I or anti-MHC II mAbs. The CD40, CD70, CD80, CD86, MHC I and MHC II expression levels were examined by flow cytometry and were evaluated on (A) $CD11c^+$ or (B) $CD11c^+CD8\alpha^+$ gated cells. Data represent one of three independent experiments.

(Figure 3A) and the splenic DCs (Figure 3B) that had been stimulated with HK vaccine expressed high levels of MHC and co-stimulatory molecules such as CD40 and CD86, as compared with the negative control. Our results revealed that, after co-culture with HK vaccine, DCs up-regulated the expression of MHC and co-stimulatory molecules such as CD40 and CD86 but not CD70 or CD80. The interaction between CD40L on activated $CD4^+$ T cells and CD40 on DCs represents an important pathway by which $CD4^+$ T cells promote $CD8^+$ T cell responses (Mackey et al. 1998; Ridge et al. 1998; Taraban et al. 2004; Van Deusen et al. 2010). Several lines of evidence have indicated that up-regulation of CD80 and CD86 and consequently engagement of the CD28 pathway are not sufficient to account for the stimulation of $CD8^+$ T cells in response to CD40 signaling (Taraban et al. 2004; Bullock and Yagita 2005). In addition, the expression of CD70 on DCs has been reported to be an important determinant for the helper-dependence of primary $CD8^+$ T cell expansion, which may explain the fact that a variety of pathogens are capable of stimulating primary $CD8^+$ T cell responses in the absence of $CD4^+$ T cells (Bullock and Yagita 2005). Therefore, we could infer that the activation of $CD8^+$ T cells ex vivo after injection of HK vaccine might be dependent on $CD4^+$ T cells. Taken together, these data indicated that HK vaccine can function as potent adjuvant to induce adaptive immunity.

### *HK vaccine induces up-regulation of CCR7 on DCs*

After capturing antigens, DCs must migrate to secondary lymphoid organs such as the spleen and lymph nodes to meet and prime antigen-specific T cells (Riol-Blanco and Sancnez-Sanchez 2005). This migration is mediated by down-regulation of the C-C chemokine receptor (CCR) 5 and up-regulation of CCR7, which enhances the capacity of DCs to migrate from the peripheral tissues to the draining lymph node (Iwasaki and Medzhitov 2004; van Duin et al. 2006; McDonnell et al. 2010). To evaluate whether HK vaccine was able to facilitate the migratory capacity of DCs, we next examined the expression levels of CCR5 and CCR7 on Flt3L-cultured BMDC and splenic DCs upon stimulation with HK vaccine. We found that both in vitro cultured BMDCs and in vivo primary DCs down-regulated CCR5, but up-regulated CCR7 when co-cultured with HK vaccine (Figure 4A).

One study using yet40 mice demonstrated that the activation of IL12p40 marked immigrant DC populations that were particularly effective in entering lymph nodes and activating naive $CD4^+$ T cells (Reinhardt et al. 2006). Since HK vaccine increased IL12p40 and CCR7 expression in DCs, we wanted to determine whether DCs stimulated with HK vaccine could migrate to the draining lymph node. After stimulation of CFSE-labeled BMDCs with CpG, HK vaccine, or medium only, cells were injected into the footpads of syngeneic mice. On day 2, the draining lymph nodes were isolated and the total number of $CFSE^+$ cells was calculated by flow cytometric analysis. We found that DCs stimulated with HK vaccine could migrate to the draining lymph node as efficiently as DCs stimulated with CpG (Figure 4B).

## Discussion

Here, we have investigated how HK vaccines elicit activation of DCs in mice. Immunization of mice with HK vaccines could stimulate DCs to induce IL12p40 expression and at the same time promote the expression of MHC and co-stimulatory molecules. In addition, HK vaccines help DCs to migrate into secondary lymphoid organs by changing the expression levels of chemokine receptors. These findings demonstrate that HK vaccines exhibit adjuvant activity.

There are functionally distinct subsets of DCs that display different capacities for cytokine production and cross-presentation (Shortman et al. 2009). For example, $CD8\alpha^+$ DCs are especially adept at activating $CD8^+$ T cells to produce cytotoxic T cells, whereas $CD8\alpha^-$ DCs are more adept at activating $CD4^+$ T cells (Villadangos and Schnorrer 2007). Interestingly, IL12p40 expression upon stimulation with HK vaccines appeared mainly in $CD8\alpha^+CD11c^+CD11b^-$ lymphoid DCs (Figure 2). This is consistent with a previous study in which $CD8^+$ DCs induced both Th1 responses and cross-primed $CD8^+$ T cells. Lymphoid DCs can produce large amounts of IL12, whereas $CD8^-$ DCs do not have this capacity. Previously, it was reported that heat-treated tumor cells had an anti-tumor effect by induction of heat shock protein (HSP) 70 (Melcher et al. 1998; Shi et al. 2006). Thus, it might be speculated that the HK vaccine elicited anti-tumor activity similarly by inducing heat shock proteins such as HSP70. However, it is unlikely that HK vaccines contained large amounts of heat shock proteins, simply because there was not enough time to induce heat shock proteins in tumor cells during the preparation of HK vaccines. As expected, HSP70 proteins could not be detected in HK vaccines by immunoblotting with anti-HSP70 antibodies (Yoon et al. 2008). Finally, it remains possible that DCs respond to HK vaccines by a novel innate immune receptor.

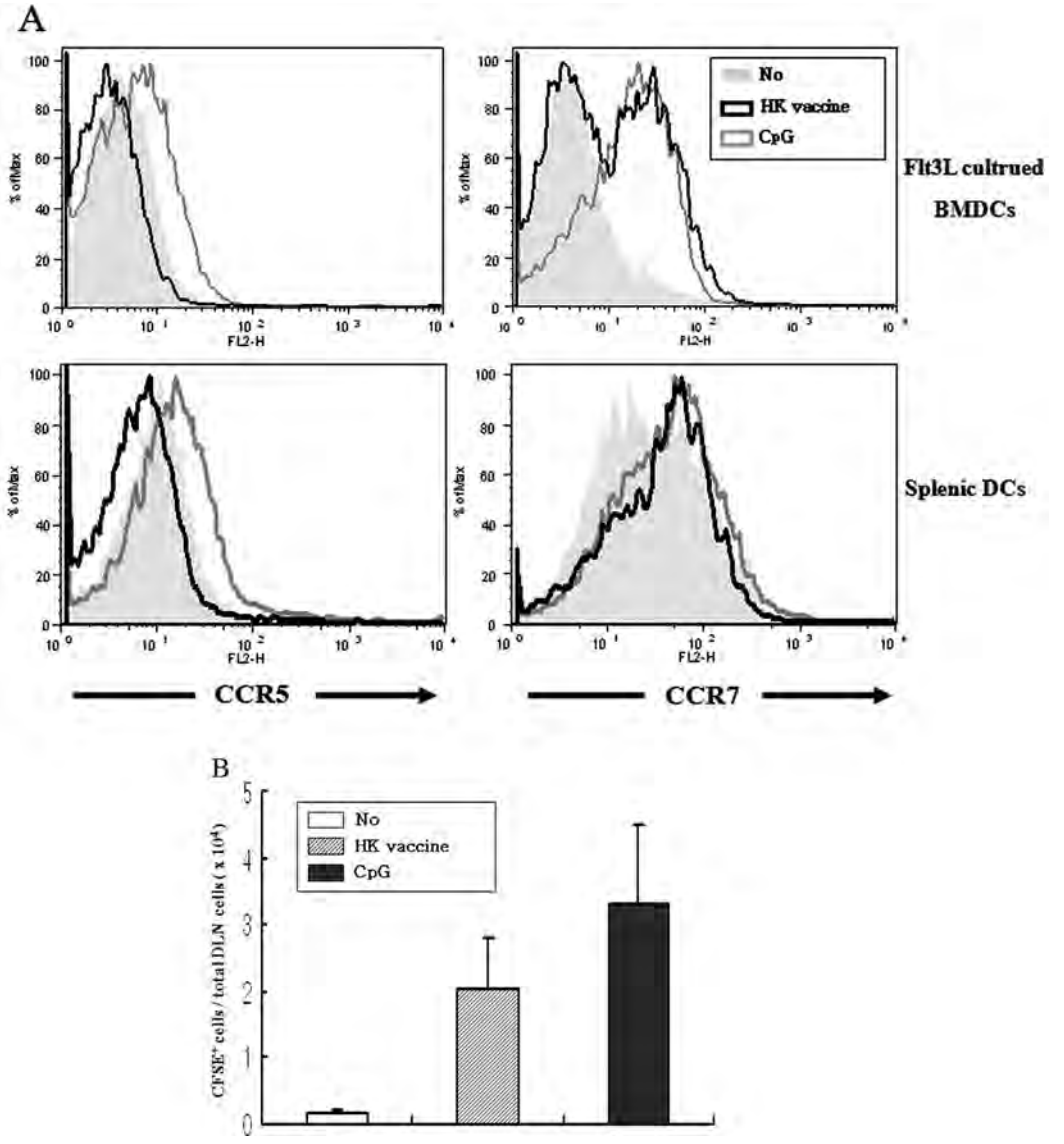


Figure 4. HK vaccines increase the capacity of DC to migrate to draining lymph nodes. (A) Flt3L-cultured BMDCs or splenic DCs were incubated with CpG (5 μg/ml), HK vaccines, or medium only for 16 hrs. These cells were stained with anti-CD11c, anti-CD8α, anti-CCR5 or anti-CCR7. CCR5 and CCR7 expression levels were evalulated on $CD11c^+$ (upper) or $CD11c^+CD8\alpha^+$ (bottom) gated cells. Data represent one of three independent experiments. (B) Flt3L-cultured BMDCs were labeled with CFSE before stimulating for 16 hrs with CpG (5 μg/ml), or HK vaccines, or medium only. After stimulation, cells ($1 \times 10^6$) were injected s.c. into the footpads of syngeneic mice. The total draining lymph nodes were isolated on day 2 and the total number of $CFSE^+$ cells was measured by using flow cytometry. Data shown represent the average $\pm$ SD ($n = 2$).

HK vaccines are basically tumor cells that are killed by heat treatment. HK vaccines maintain the shape of cells for an extended time period, although they are no longer viable. From a clinical perspective, HK vaccines have two advantages over current tumor vaccines listed in the literature (Schuster et al. 2006). First, it is very easy to prepare HK vaccines directly from tumor cell cultures because of the simplicity of the protocol. Second, HK vaccines are safer than most other tumor vaccines because they can be generated without any toxic treatment from tumor cells derived from patients. Therefore, it will be more appropriate to use HK vaccine as a therapeutic rather than a preventive vaccine for treating cancer patients.

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