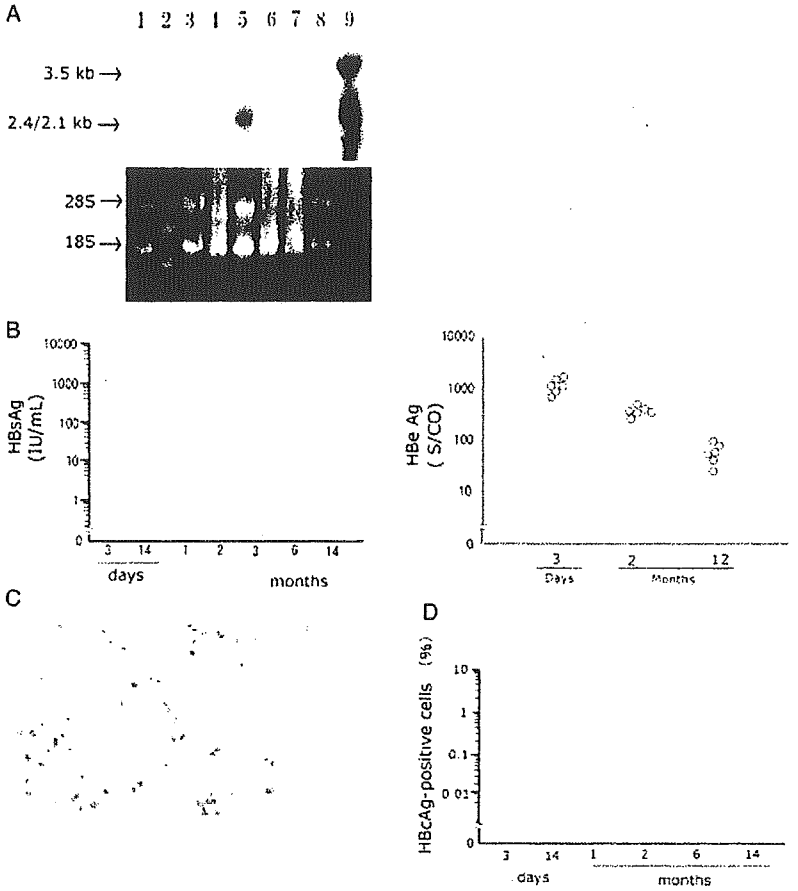

Fig. 2. Expression of HBV in hydrodynamically-transfected nude mice. (A) HBV RNA expression in various organs. Total RNA isolated from the indicated organs was analyzed for the presence of HBV-specific sequence 3 days after transfection (upper panel). Huh7 transfected with pHBV1.5 or pCMV were included as positive and negative controls, respectively. Arrows indicate 3.5- and 2.4/2.1-kb transcripts. A photograph of the ethidium bromide-stained gel is also shown in the lower panel. Lane 1, brain; lane 2, heart; lane 3, thymus; lane 4, lung; lane 5, liver; lane 6, kidney; lane 7, spleen; lane 8, pCMV-transfected Huh7; lane 9, pHBV1.5-transfected Huh7. (B) HBsAg and HBeAg in serum. The levels of HBsAg and HBeAg were serially determined in a cohort of mice hydrodynamically transfected with pHBV1.5. (C) Immunohistochemical detection of HBc Ag. Representative data for nude mice 3 days after pHBV1.5 injection. (D) Frequency of HBcAg-positive hepatocytes in the livers.

To examine the kinetics of viremia, we examined the levels of DNase I-resistant HBV DNA in serum by real-time PCR analysis (Fig. 3B). The levels of HBV DNA were as high as 1×10^7 copies/ml at 3 days after the injection and gradually decreased by 1.5 log over 1 year.

3.3. Long-term expression of HBV is dependent on HBV replication

The extremely long-term expression and carriage of HBV in this system led us to examine whether episomal replication could affect the kinetics of expression of HBV-related genes. Toward this goal, we introduced point mutation in the *pol* gene of pHBV1.5 which could produce the truncated form of the HBV polymerase without affecting the expression of any other HBV-related proteins. Mice hydrodynamically injected with mutant pHBV1.5 produced HBsAg as well as HBcAg at levels similar to those of wild-type pHBV1.5-injected mice 3 days after injection (Fig. 4A and B). However, mutant pHBV1.5-induced expression of HBsAg, HBeAg and HBcAg was terminated within 2 months, in striking contrast to wild-type pHBV1.5-induced gene expression (Fig. 1B and D). Northern blot analysis confirmed the transient expression of HBV genes after injection of mutant pHBV1.5 (Fig. 4C).

HBV DNA polymerase binds to the 5′ end of its own mRNA template, and the complex is then packaged into nucleocapsids, where viral DNA synthesis occurs [19]. HBV genomic DNA produced via the reverse transcription pathway predominantly consists of relaxed-circular DNA with a complete minus strand and a partially synthesized plus strand. In natural HBV infection in humans, part of the nucleocapsids migrates to the nucleus where relaxed-circular DNA is converted to cccDNA that serves as a template for transcription [19]. The finding in the present model of long-term expression of HBV involving HBV DNA replication suggested that viral cccDNA may be produced in murine livers and work as a transcriptional

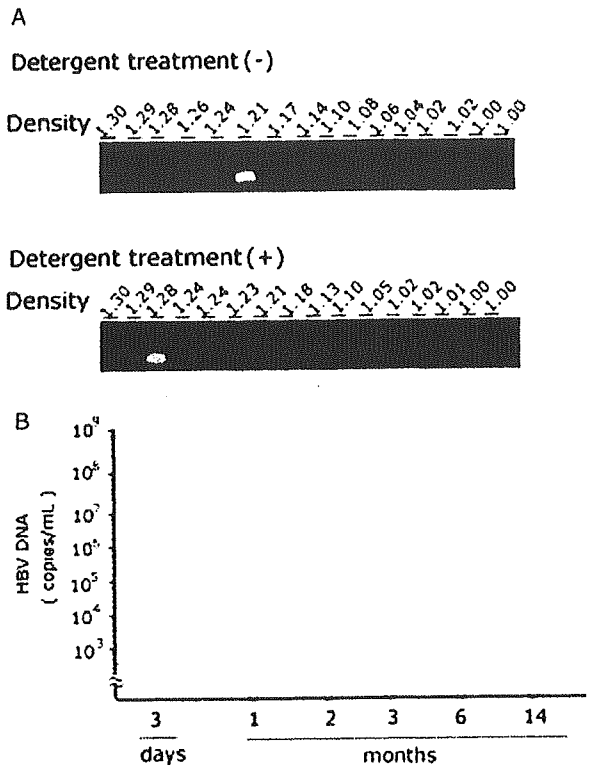

Fig. 3. Production of HBV in hydrodynamically-transfected mice. (A) Density of HBV. Sera from mice injected 3 days earlier with pHBV1.5 were treated with or without *detergent* then fractionated through sucrose density gradient. DNA was isolated from each fraction after treatment with DNase I and then the presence of the HBV DNA sequence was checked by PCR. The density (g/cm^3) of each fraction is indicated above each lane. (B) Viral titers in serum. The levels of HBV DNA were serially determined by real-time PCR.

template for HBV expression, in addition to the inoculated plasmid. To examine the presence of cccDNA in the liver, we used a PCR procedure which selectively detects cccDNA (Fig. 1). We also checked for the presence of inoculated plasmids by amplifying the ampicillin resistance gene by PCR. The authenticity of the cccDNA detection was confirmed by the detection of a specific signal from liver tissues of patients with chronic hepatitis B, but not from the serum of patients or pHBV1.5 (Fig. 5A). Viral cccDNA was clearly detected in wild-type pHBV1.5-injected livers at 3 days as well as 3 months after the injection (Fig. 5B). As expected, cccDNA was not detected in mutant pHBV1.5-injected livers. The levels of cccDNA were measured by real-time PCR (n=5 for each time point) and results were 2.4×10^7 and 6.0×10^5 copies per gram of liver tissue at 3 days and 2 months after the injection, respectively. Since the liver approximately contains 1.1×10^8 of hepatocytes, the average copy numbers of HBV cccDNA per core Ag-positive hepatocyte could be estimated to be 1 or 4. Ampicillin resistance gene was similarly amplified from both wild-type pHBV1.5- and mutant pHBV1.5-injected livers. The fact that HBV gene expression was terminated within 2 months upon injection of mutant pHBV1.5 clearly indicates that the presence of residual plasmids in the livers at later time points is not sufficient for the expression of detectable levels of HBV genes; this is consistent with a previous report [20] demonstrating that transgene expression is rapidly terminated after hydrodynamic gene delivery despite the persistence of plasmid DNA in the livers. These results support the idea that viral cccDNA is critically involved in the long-term expression and carriage of HBV in this model.

3.4. Administration of IFNα gene transiently suppressed HBV DNA replication and failed to eradicate viral template

We next sought to examine the potential usefulness of this model for the assessment of anti-viral drugs. To examine the effect of IFNα in the phase of cccDNA-dependent HBV replication, we injected either pCMV-IFNa1 or pCMV at 70 days after pHBV1.5 injection. Injection of pCMV-IFNa1 led to substantial IFNα production at day 1 (Fig. 6A), although IFNα could not be detected in the mock-injected mice (data not shown). The levels of IFNα after pCMV-IFNa1 injection rapidly declined at day 3 and could not be detected at day 28. Injection of pCMV-IFNa1 significantly suppressed viral production at day 3 but did not affect HBs production (Fig. 6B and C); this is consistent with previous findings [15,21] that IFNα suppressed HBV replication at a step of reverse transcription. In spite of the substantial suppression of HBV production at day 3, the levels of viral titers of mice injected with pCMV-IFNa1 increased to levels similar to those of pCMV-injected mice at day 14 and later. These results indicate that IFN treatment substantially suppressed viral replication, but could not eliminate the viral template from the infected host. This model should be useful for assessing anti-viral therapy aimed at eradication of the viral template.

4. Discussion

In the present study, we demonstrated that hydrodynamic injection of a plasmid encoding an overlength of HBV DNA into nude mice established long-term replication of HBV in the liver. Since hepatic damage was not observed, this model mimics the chronic carrier-like state of human HBV infections. This model reminds us of a 1988 report by Feitelson et al., [22] in which they stated that intrahepatic injection of replication competent HBV DNA led to persistent HBs antigenemia as well as chronic liver injury in nude mice. They had no evidence of HBV replication such as production of Dane particles in the circulation. In a preliminary experiment, we intrahepatically injected pHBV1.5 into nude mice and monitored viral production in the serum. DNase I-resistant HBV DNA could not be detected in most mice tested; a small number of mice

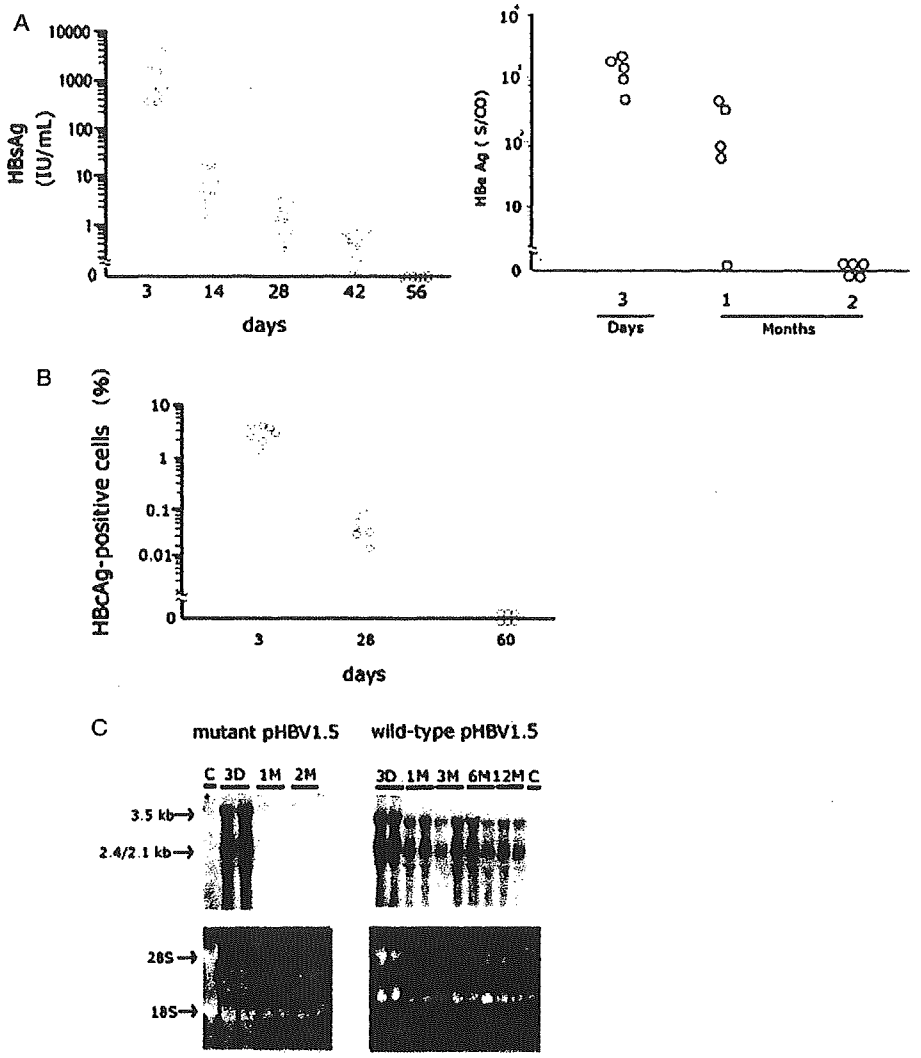

Fig. 4. Expression of HBV genes in nude mice injected with replication-incompetent pHBV1.5. (A) Serial detection of HBsAg and HBeAg in serum. (B) Frequency of HBcAg-positive hepatocytes in the livers. (C) Time-course of HBV RNA expression in the livers transfected with mutant pHBV1.5 or wild-type pHBV1.5 determined by Northern blot. C, control livers; 3D, 1M, 3M, 6M, and 12M, liver samples obtained at 3 days, 1 month, 3 , 6 , and 12 months after hydrodynamic injection, respectively.

produced low levels of virus at 3 days after injection but not at later time points (our unpublished data). Thus, the transfection efficiency of hydrodynamic injection of HBV DNA appeared to be higher than that of intrahepatic injection. Despite the difference in liver damage observed among these studies, we considered the absence of hepatitis in the present model reasonable, since the T-cell immune response towards HBV-related antigens could not occur. Furthermore, it should be noted that the antigenemia as well as HBV production achieved by the hydrodynamic procedure was very reproducible, which is critically important when applying this model to evaluate the efficacy of anti-viral drugs.

The duration of hydrodynamics-based gene expression varies among reports from days to months [19,23,24]. The plasmid-based gene expression of our model terminated within 2 months, as demonstrated by the injection of replication-incompetent HBV DNA (mutant pHBV1.5). Replication-competent HBV DNA (wild-type pHBV1.5)-injected mice displayed a rapid decline of HBsAg production followed by relatively stable antigenemia for more than 1 year (Fig. 2B). Although the rapid decline observed in the first 2 weeks may reflect the plasmid-based gene expression, stable expression of HBsAg at later time points did not depend on residual plasmids in the livers, but required intracellular reproduction of HBV DNA. These results indicate that HBV replication in addition to immunological tolerance is critically important for long-term HBV expression in this system. Previous research on in vivo gene transfer [8] and transgenic mice [5] has indicated that HBV cccDNA, the template of HBV replication in natural infection, could not be detected in

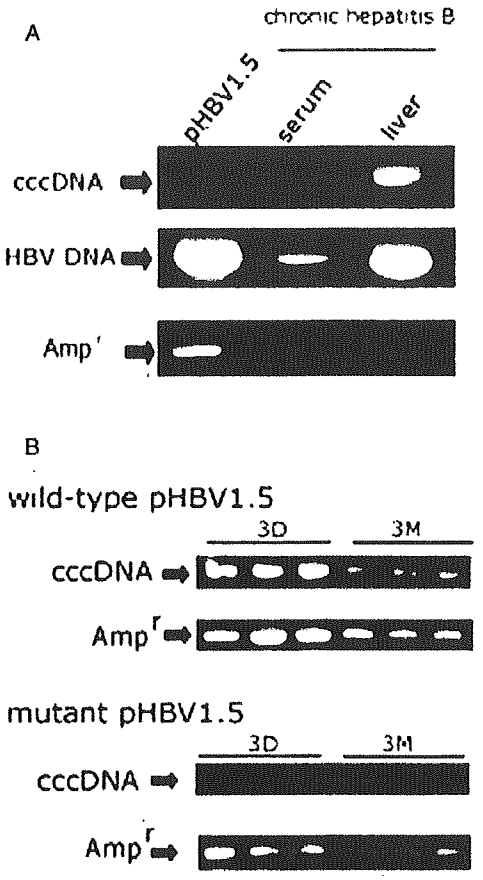

Fig. 5. Detection of HBV cccDNA and ampicilin resistance gene by PCR. (A) Specificity of HBV cccDNA detection by PCR. DNA were isolated from the following samples and amplified for the detection of cccDNA, ampicilin resistance gene, and HBV DNA sequence by corresponding PCRs. pHBV1.5, sample containing pHBV1.5; serum, serum from a patient with chronic hepatitis B; liver, a liver specimen from the same patient. (B) DNA was isolated from the liver samples 3 days (3D) or 3 months (3 M) after hydrodynamic injection of either wild-type pHBV1.5 or mutant pHBV1.5 ($N=3$ for each group) and examined the presence of cccDNA and ampicilin resistance gene by PCRs.

murine livers by Southern blot analysis. In the present study, we applied a highly sensitive PCR procedure and detected HBV cccDNA in pHBV1.5-injected livers. What is important is that the estimated numbers of HBV cccDNA per hepatocyte were 1 or 4, which should be sufficient for HBV gene expression. Taken together, the present study is the first demonstration of the production of viral cccDNA and its contribution to HBV replication in mice. Thus, the species restriction on the production of HBV cccDNA may not be as strict as has previously been believed.

Mutation of HBV DNA occurring during therapy with various nucleotide analogues leads to drug resistance and limits the success of these drugs for controlling HBV replication in humans [1,2]. Thus far, except for the in vitro recombinant HBV baculovirus system [25], there has been no useful model supporting reproduction of the HBV viral

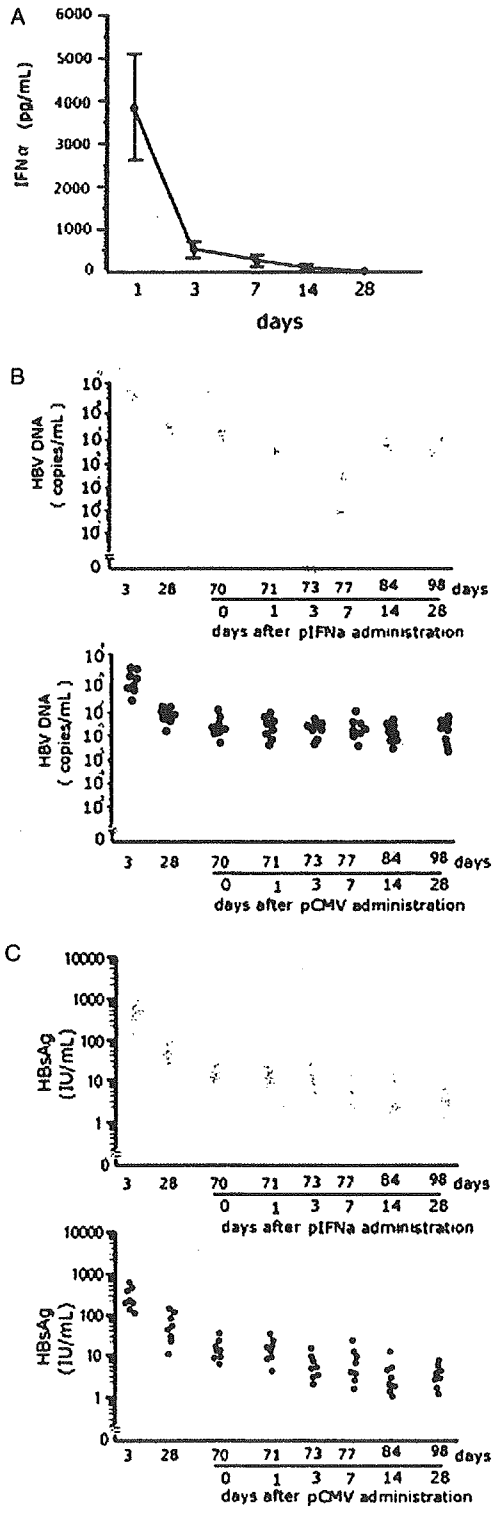

Fig. 6. Transient suppression of HBV production by IFNα gene therapy. (A) Serial determination of serum IFNα levels of nude mice after injection of pCMV-IFNa1. Horizontal bars indicate SD. (B and C) Nude mice were transfected with pHBV1.5 and, at 70 days later, transfected with either pCMV-IFNa1 (open circles) or pCMV (closed circles). Sera were serially obtained from the retro-orbital plexus, with HBV DNA (B) and HBsAg (C) titers being determined.

template as is the case of hepatitis C virus replicon systems [26,27]. Although HBV could not 'infect' murine hepatocytes, intracellular 'reinfection', namely recycling of HBV DNA occurs and leads to chronic viral production in the present model. Therefore, this model may provide a unique opportunity for analyzing possible mutations induced by long-term usage of various nucleotide analogs. Further study is needed to examine this possibility. Finally, intentional mutation could be easily introduced in inoculated DNA and a wide variety of mice with different genetic backgrounds can be used. The model presented here should enable analysis of viral as well as host factors that may regulate HBV replication.

Acknowledgements

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Quick Generation of Fully Mature Dendritic Cells From Monocytes With OK432, Low-Dose Prostanoid, and Interferon-α as Potent Immune Enhancers

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Summary: Dendritic cells (DCs) are one of the promising tools for enhancing antigen-specific immune responses in clinical settings. Many studies have been performed thus far to verify the efficacy of the DC vaccine in cancer patients; however, the responses have not always been satisfactory, partly because of DC incompetence. To obtain DCs potentially applicable for vaccination of cancer patients, our group sought to establish the strategy of DC generation mainly by modulating culture periods and maturation stimuli. Novel mature DCs that can be generated from monocytes within 3 days by using a combination of OK432 (*Streptococcus pyogenes* preparation), low-dose prostaglandin E_2 (PGE_2), and interferon-α (OPA-DCs) were developed. They strongly express CD83, CD86, and CCR7 and have potent ability to migrate to CCL21. In addition, they were able to activate natural killer and T helper 1 (T_H1) cells and to induce peptide-antigen–specific cytotoxic T lymphocytes more significantly than monocyte-derived DCs stimulated with a conventional cytokine cocktail of tumor necrosis factor-α, interleukin (IL)-1β, IL-6, and PGE_2 (monocyte-conditioned medium [MCM]-mimic DCs). The profound ability of OPA-DCs to stimulate these effectors is attributable to their higher expression of IL-12p70, IL-23, and IL-27 than MCM-mimic DCs, which was supported by the findings that the neutralization of IL-12p70 and IL-23 reduced the T_H1 priming ability of OPA-DCs. Even when from advanced gastric or colonic cancer patients, OPA-DCs displayed abilities of migration and T_H1 induction comparable to those from healthy subjects. Therefore, OPA-DCs may serve as a feasible vaccine with the potential to enhance T_H1-dominant and cytolytic immune responses against cancers.

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Informed consent was obtained from all the volunteers for the completion of this study.

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Key Words: dendritic cells, cancer immune therapy, OK432, prostaglandin E_2, interferon-α

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Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) that play a central role in innate and acquired immunity. For the treatment of cancers, tumor antigen-loaded DCs have been considered as a therapeutic vaccine to induce tumor-specific immunity. Many clinical studies have been performed to assess the efficacy of DC vaccine against cancers; however, favorable immunologic outcomes have been obtained from only half of the vaccinated patients.[1] Overall, the lessons from these studies are that mature DCs are better than immature ones to induce anticancer immune responses in the vaccinated patients.

Although the protocols of mature DC generation are yet to be standardized, a monocyte-conditioned medium (MCM)-mimic is widely used as a maturation stimulus for monocyte-derived DCs (MoDCs). The MCM-mimic is a combination of recombinant cytokines first reported by Jonuleit et al.[2] It gives rise to mature DCs in vitro; however, less than 10% of cancer patients vaccinated with MCM-mimic–treated DCs displayed favorable clinical responses (partial or complete remission).[3–5] These observations suggest that the MCM-mimic may fall short of generating mature DCs capable of inducing an in vivo immune response. One of the reasons may be that the MCM-mimic lacks the ability to promote DCs to secrete interleukin (IL)-12p70,[6] which is well known as an enhancer of cytotoxic activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs).[7,8] Therefore, it may be necessary to improve the maturation stimuli of DCs with respect to the functional requirements of the DC vaccine. From a mechanistic point of view, DCs loaded with antigens migrate into draining lymph nodes (DLs), where they activate NK cells or present antigens to $CD4^+$ and $CD8^+$ T cells.[9,10] Thus, to induce potent antitumor immunity, DCs need to possess the abilities to migrate and stimulate these effectors, which has been demonstrated in some murine models.[11–14]

Recently, other investigators have demonstrated that monocytes differentiate into mature DCs in 2 days when an

MCM-mimic is used.[15] It has also been reported that OK432, a penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus pyogenes*, provides MoDCs with the ability to induce a T helper 1 (T_H1) response.[16,17] In the present study, we sought to establish functionally mature MoDCs mainly by modulating culture periods and maturation stimuli. Using OK432 in combination with prostaglandin E_2 (PGE_2) and interferon (IFN)-α, we successfully generated mature DCs in only 3 days. In comparison with the MCM-mimic, this cocktail enables DCs to gain more potent abilities in migration, IL-12p70 secretion, NK cell activation, T_H1 induction, and CTL generation. These novel quickly generated DCs are a promising tool for developing DC vaccines against cancers.

MATERIALS AND METHODS

Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human IL-7 and IL-2 were obtained from Genzyme-Techne (Minneapolis, MN). Recombinant human tumor necrosis factor (TNF)-α, IL-6, and IL-1β were purchased from R&D Systems (McKinley Place, NE). OK432 (Picibanil) was kindly provided by Chugai Pharmaceutical Company (Tokyo, Japan). The amount of OK432 is expressed in units designated as KE (Klinische Einheit [clinical unit]). One KE OK432 is equivalent to 0.1 mg dry streptococci. Natural human IFNα was kindly provided by Otsuka Pharmaceutical Company (Tokyo, Japan). PGE_2 was purchased from Sigma (St. Louis, MO). Nine-mer peptide carcinoembryonic antigen (CEA) 652(9) (TYACFVSNL), reported to be a human leukocyte antigen (HLA)-A24 restricted CTL epitope in CEA,[18] was purchased from TaKaRa Bio (Shiga, Japan).

Cell Lines

T2-A24 is a transporter associated with an antigen processing (TAP) deficient cell line (T2) transfected with HLA-A*2402 gene. This cell line expresses a high level of HLA-A24 protein and is used for targets in cytotoxicity assay (a kind gift from Dr. Hideaki Tahara, University of Tokyo, Tokyo, Japan). T2-A24 and NK cell-sensitive cell line K562 were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2.

Preparation of Various Dendritic Cells From Monocytes

Buffy coat from healthy blood donors was kindly provided by the Red Cross Blood Center (Osaka, Japan). After written informed consent had been obtained, blood samples were collected from healthy donors or patients with primary untreated and advanced gastric or colonic cancer followed at Osaka University Hospital, Osaka Police Hospital, or Saiseikai Senri Hospital.

Peripheral blood mononuclear cells (PBMCs) were separated from the buffy coat or fresh peripheral blood from donors by standard density gradient centrifugation using Lymphocyte Separation Solution (Nacalai Tesque, Kyoto, Japan). Monocytes were isolated from PBMCs using anti-CD14 microbeads (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. The purity of the $CD14^+$ cells verified by flow cytometry was 90% to 95%.

Quickly Induced and Standard Immature Dendritic Cells

Monocytes were seeded on 24-well culture plates (Falcon, Franklin Lakes, NJ) at 5×10^5 per well and cultured in serum-free AIM-V media (Invitrogen, Carlsbad, CA) containing 50 ng/mL GM-CSF and 20 ng/mL IL-4. Nonadherent cells harvested on day 3 of culture were called quickly induced immature DCs (qiDCs). Half of the culture supernatants of the remaining cells were replaced with AIM-V media containing the same amount of GM-CSF and IL-4 on day 4. Subsequently, nonadherent cells of each group were harvested on day 7 and called standard immature DCs (siDCs).

Standard Mature Dendritic Cells

For the generation of standard mature DCs (smDCs), monocytes were cultured as for siDCs. During the final 24 hours, the cells were matured with 0.1 KE/mL OK432 in the presence (smDC-op) or absence (smDC-o) of 350 ng/mL PGE_2.

Quickly Induced Mature Dendritic Cells

For the generation of quickly induced mature DCs (qmDCs), monocytes were cultured as for qiDCs. During the final 24 hours, the cells were matured with 0.1 KE/mL OK432 in the presence (qmDC-op) or absence (qmDC-o) of various concentrations of PGE_2. In addition, other qmDCs were generated with some combinations of reagents or cytokines in a similar manner (combination of OK432, low-dose PGE_2, and IFNα [OPA]-DCs and MCM-mimic [MCMm]-DCs). The definitions of DCs generated in the various protocols are summarized in Figure 1.

Yield of Various Dendritic Cells

After the generation of various DCs, nonadherent cells were harvested and counted. Subsequently, the harvested cells were stained with anti-CD11c–fluorescein-isothiocyanate (FITC) monoclonal antibody (mAb) (KB90; DakoCytomation, Kyoto, Japan) and anti-HLA–D-related (DR)–phycoerythrin (PE) mAb (L243; Becton Dickinson, Franklin Lakes, NJ). The cells positive for CD11c and HLA-DR were defined as DCs. The absolute number of harvested various DCs was calculated from the percentage of DCs in the collected cells. The yield of DCs was defined as the percentage of recovered DCs in the seeded monocytes.

Analysis of Dendritic Cell Phenotype

DCs were analyzed for CD40, CD80, CD83, CD86, CCR7, CD14, and HLA-DR expression using fluorescent material–conjugated mouse mAbs. Anti-human CD40 mAb (5C3), anti-human CD80 mAb (L307.4), and anti-human CD83 mAb (HB15a) were from Immunotech (Marseille,

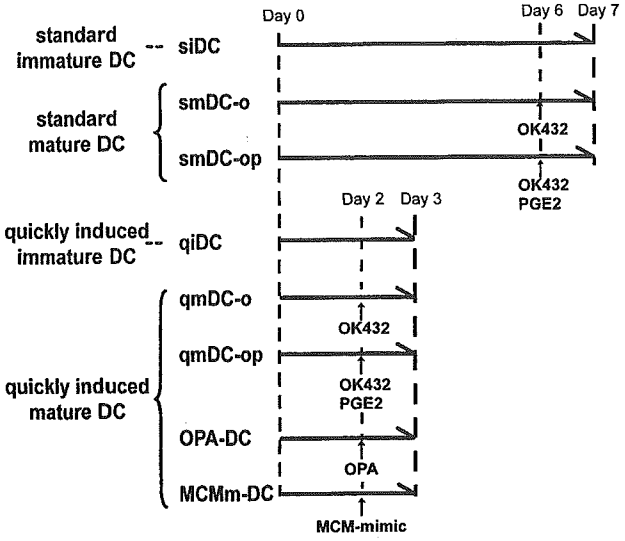


FIGURE 1. Preparation of DCs generated by means of various protocols. siDCs are generated with GM-CSF and IL-4 over 7 days. smDCs are siDCs stimulated with OK432 in the absence (smDC-o) or presence (smDC-op) of 350 ng/mL PGE_2 for the final 24 hours. qiDCs are generated from monocytes with IL-4 and GM-CSF for 3 days. qmDCs are qiDCs stimulated with OK432 combined with other reagents for the final 24 hours (qmDC-o: OK432 only, qmDC-op: OK432 and PGE_2). The concentrations of reagents used were 0.1 KE/mL OK432 and 10 to 1000 ng/mL PGE_2. In addition, qmDCs with OPA (OPA-DCs) or with MCM-mimic (MCMm-DCs) were generated for later experiments. (OPA: 0.1 KE/mL OK432, 500 IU/mL IFNα, and 50 ng/mL PGE_2; MCM-mimic: 10 ng/mL TNFα, 10 ng/mL IL-1β, 10 ng/mL IL-6, and 350 ng/mL PGE_2).

France). Anti-human CD86 mAb (BU63) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human CCR7 mAb (150503) was from R&D Systems. Anti HLA-DR mAb (L243) and anti-human CD14 mAb (MΦP9) were from Becton Dickinson. The phenotypic analysis of DCs was performed using FACSCaliber (Becton Dickinson) and Cell Quest software (Becton Dickinson).

Migration Assay

The migratory ability of DCs was examined by translocation of DCs in response to chemokine through a polycarbonate filter with 5-μm pores in 24-well transwell chambers (Corning Coster, Cambridge, MA). Into the lower chambers, 500 μL AIM-V with and without 500 ng/mL CCL21 (R&D Systems) was introduced. DCs were placed in the upper chambers at 1×10^5 per well and incubated for 2 hours at 37°C. The cells that migrated to the lower chambers were harvested and counted. The number of spontaneously migrated cells, those in the well without the addition of CCL21, was subtracted from the number of migrated cells in the well with the addition of CCL21.

Subsequently, the original cells before the migration assay and the cells migrating to the lower chamber containing CCL21 were stained with anti-CD11c-FITC mAb and anti HLA-DR-PE mAb, and the percentage of CD11c and HLA-DR double-positive cells was analyzed by flow cytometry. The percentage of migrated DCs in response to CCL21 was calculated as follows.

$$\text{Migrated DCs} = \frac{\%\text{ of double positive migrated cells} \times \text{migrated cells to CCL21}}{\%\text{ of double positive original cells} \times 1 \times 10^5} \times 100\,(\%)$$

Cytokine Measurement

Cytokine Producing Ability of Various Dendritic Cells

To test the ability of DCs to produce IL-12p70 and IL-10, DCs at a rate of 1×10^5 per well were cultured with 1×10^5 per well murine fibroblasts transfected with human CD40L (CD40L/L-cell) and 100 IU/mL IFNγ in 96-well flat-bottom plates (Asahi Techno Glass, Tokyo, Japan). After 24 hours of incubation, the culture supernatants were collected and the concentration of IL-12p70 and IL-10 in the samples was examined by means of enzyme-linked immunosorbent assay (ELISA).

Type 1 Helper T-Cell–Inducing Ability of Various Dendritic Cells

To test the ability of DCs to stimulate a T_H1 response, 1×10^5 per well allogeneic $CD4^+$ $CD45RO^-$ naive T cells were cultured with 1×10^4 per well DCs in 96-well flat-bottom plates. In some experiments, 10 ng/mL anti-human IL-23 polyclonal antibody and/or 25 ng/mL anti-human IL-12p70 mAb (24910; R&D Systems) was added to the culture for neutralization of each cytokine. Naive $CD4^+$ T cells were prepared from PBMCs by negative selection using a Stem-Sep system (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions. The purity of isolated $CD4^+$ $CD45RO^-$ naive T cells was 90% to 95%, as determined by flow cytometry. On day 4, 10 ng/mL IL-2 was added to each well. On day 7, the cells were harvested and stimulated with 10 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma) and 1 μg/mL ionomycin (Sigma). After 24 hours of incubation, the culture supernatants were collected and the concentration of IFNγ and IL-10 in the samples was examined by means of ELISA.

Paired antibodies for the detection of human IL-12p70, IFNγ, and IL-10 were purchased from Endogen (Woburn, MA). The range of the assay was 15 to 1000 pg/mL.

Quantification of p19, p40, p28, and Epstein-Barr Virus–Induced Gene 3 Messenger RNA Expression of Various Dendritic Cells

Quantitative analysis of p19, p40, p28 and Epstein-Barr virus–induced gene 3 (EBI3) messenger RNA (mRNA) expression was performed in various DCs using real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from DCs using an RNeasy mini kit (Qiagen, Valencia, CA). First-strand complementary DNA (cDNA) was synthesized from the total RNA with the Super Script III First-Strand Synthesis System (Invitrogen).

Quantification of the p19, p40, p28, EBI3, and β-actin transcripts was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the TaqMan probe method. The reaction protocol was identical for all PCR products. In brief, a 2-minute incubation at 50°C and a 10-minute incubation at 95°C were followed by 40 cycles of sequential incubations at 95°C (15 seconds) and 60°C (1 minute) for data collection. The β-actin mRNA expression of all samples was quantified as an endogenous standard, and normalization to the β-actin was performed for each sample.

Analysis of Natural Killer Stimulatory Activity of Various Dendritic Cells

The ability of DCs to stimulate autologous NK cells was assessed by the cytotoxicity of NK cells. NK cells were prepared from $CD14^-$ cells by positive selection using anti-CD56 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated $CD56^+$ NK cells was 90% to 95%, as determined by flow cytometry. Various DCs were cultured with autologous NK cells in 24-well culture plates (Falcon) for 24 hours at 37°C in 5% CO_2. Subsequently, these cells were cultured with K562 cells labeled with $Na_2{}^{51}CrO_4$ at various effector/target (E/T) ratios for 4 hours at 37°C in 5% CO_2. For spontaneous release, target cells were incubated with medium alone, and for maximum release, target cells were incubated with medium containing 10% Triton X-100 (Sigma). Supernatants were then harvested, and radioactivity was counted with a Wizard 3 gamma counter (Wallac, Boston, MA). Percentages of target cell lysis were calculated as follows.

$$\text{Specific lysis} = \frac{\text{sample release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\ (\%)$$

Induction of Carcinoembryonic Antigen–Peptide–Specific Cytotoxic T Cells With Various Dendritic Cells

CTLs specific for CEA 652(9) peptide were generated according to a method described previously.[19] After informed consent had been obtained, fresh blood samples were taken from HLA-A24–positive healthy donors. As for responder cells, $CD14^-$, $CD19^-$, and $CD56^-$ cells were isolated by depleting $CD19^+$ and $CD56^+$ cells from $CD14^-$ cells using anti-CD19 and anti-CD56 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. After various DCs were generated as described previously, they were incubated with 20 μg/mL CEA.652(9) peptide for 6 hours at 37°C. Subsequently, they were cultured with autologous $CD14^-$, $CD19^-$, and $CD56^-$ cells in DC medium (DCM) for 28 days at 37°C in 5% CO_2. The DCM is the Iscove modified Dulbecco medium (Invitrogen) containing 10% FCS, 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Invitrogen), 100 μM nonessential amino acid (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin. On days 7, 14, and 21, the same numbers of peptide-loaded freshly prepared DCs from the same donor were supplied to the culture as stimulators. Recombinant human IL-7 was added at 5 ng/mL on days 1 and 7. In addition, recombinant human IL-2 was added at 5 ng/mL every 3 days from day 10 of CTL induction.

Analysis of Carcinoembryonic Antigen–Peptide–Specific Cytotoxic T-Cell Activity

CEA-peptide–specific cytotoxic T cells were induced with various DCs as mentioned previously. On day 28 of CTL induction, the cells were harvested. Before analysis of cytotoxicity, the remaining $CD56^+$ cells were removed from the DC-primed cells with anti-CD56 microbeads (Miltenyi Biotec). The lytic activity of CTL against CEA 652(9) peptide-loaded T2-A24 was assessed by means of a ^{51}Cr releasing assay. To confirm that the lytic activity is exerted in an HLA class I-restricted and CD8-restricted manner, 10 μg/mL mouse monoclonal anti-HLA–ABC antibody (W6/32) (Serotec, Oxford, UK) or mouse monoclonal anti-human CD8 antibody (DK25) (DakoCytomation) was added to the mixture of effectors and targets. To exclude the possibility of the lytic activity being mediated by NK cells, K562 was also used as a target cell in the assay.

Statistical Analysis

The results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was analyzed using the paired 2-tailed *t* test with Prism 4 software (GraphPad Software, San Diego, CA). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Mature Dendritic Cells Can Be Quickly Induced by OK432

Other investigators have reported that monocytes differentiate into DCs even within 48 hours.[15] Quick generation of DCs is beneficial for the maintenance of their cell viability. Thus, to examine whether OK432 can induce mature DCs in a shorter culture period, we added it to the culture on day 2. Although all DCs were positive for HLA-DR but negative for CD14, qiDCs expressed lesser degrees of CD80, CD83, and CD86 than siDCs. OK432 enhanced the expression of costimulatory factors (CD40, CD80, CD83, and CD86) on qmDC-o as well as on smDC-o, however (Fig. 2). Thus, the addition of OK432 to DCs at an early phase induced phenotypically mature DCs in a short culture period.

Combination of OK432 and Prostaglandin E_2 Quickly Induces Mature Dendritic Cells With Potent Migratory Ability

To compare the migratory capability of DCs generated by quick or standard generation protocols, we examined CCR7 expression and the migration of DCs to CCL21 in a transwell

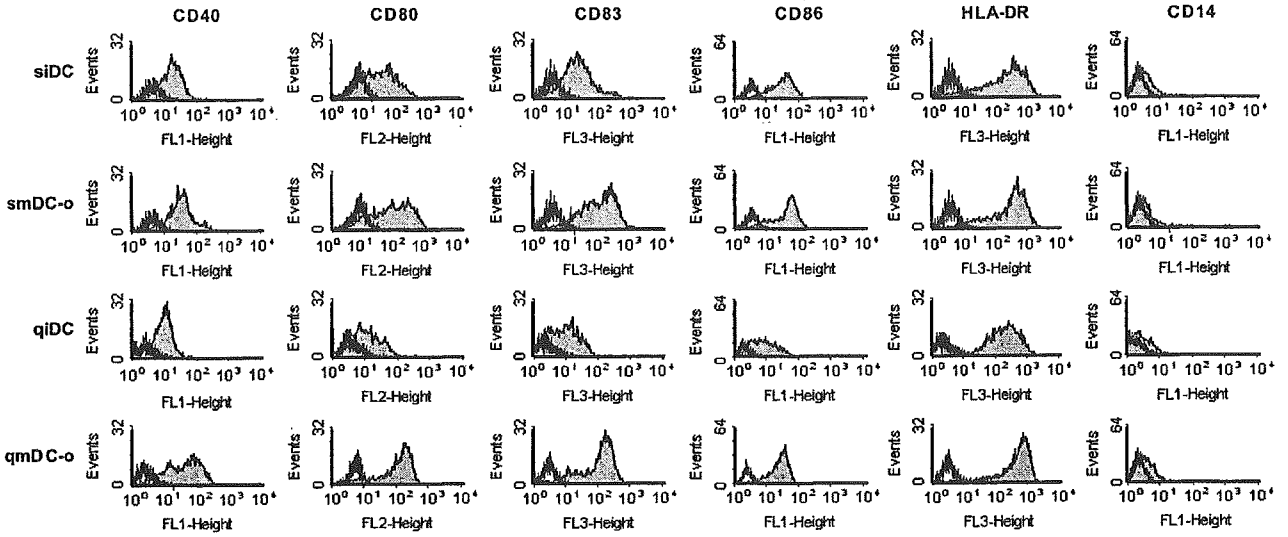


FIGURE 2. OK432 induces maturation on qiDCs and siDCs. Cells differentiated under distinct culture conditions were analyzed by flow cytometry for the expression of CD40, CD80, CD83, CD86, HLA-DR, and CD14 (thin lines with a shaded portion). Thick lines with an unshaded portion show the results of cells without staining. Various DCs were generated, as described in Fig. 1.

system (Fig. 3). CCR7 expression and the migrating ability of DCs were slightly enhanced with the addition of OK432 alone to qmDC-o and smDC-o compared with those without. Recently, Scandella et al[20] reported that PGE_2 is an important factor for enhancing the migratory capacity of MoDCs toward CCL21. Therefore, to induce more potent migrating ability of DCs, we added PGE_2 combined with OK432 to qiDCs or siDCs. We found that the combination of OK432 and PGE_2 upregulated CCR7 expression on DCs more than OK432 alone (smDC-op and qmDC-op). In parallel with CCR7 expression, DCs cultured with the combination of OK432 and PGE_2 showed significantly higher migratory potential toward CCL21 than those cultured with OK432 alone on qiDCs and smDCs. Interestingly, the qmDC-op generated in only 3 days showed more significant migration than the smDC-op generated in 7 days.

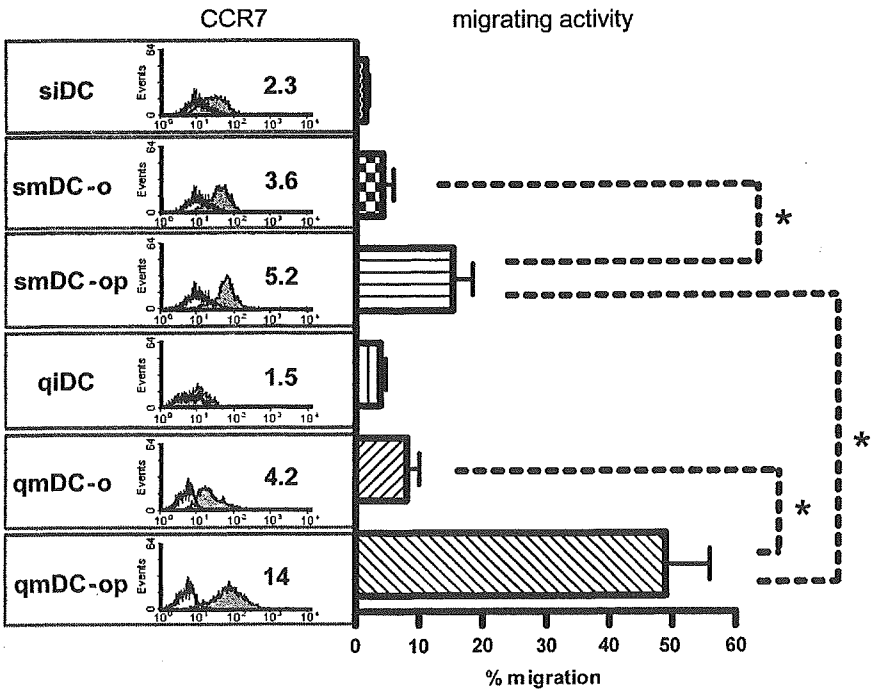


FIGURE 3. PGE_2 combined with OK432 enhances CCR7 expression and migration to CCL21 of qiDCs and siDCs. Various DCs were generated, as described in Fig. 1. They were analyzed by flow cytometry for the expression of CCR7. Thin lines with a shaded portion show CCR7 expression of various DCs. Thick lines with an unshaded portion show the results of various DCs without staining. The numbers indicate the ratios between the mean fluorescence intensity of each sample with and without staining. In addition, the migratory potential of various DCs toward CCL21 was analyzed, as described in the Materials and Methods section. The results are expressed as the mean ± SEM of 5 experiments ($*P < 0.05$).

Combination of OK432, Interferon-α, and Low-Dose Prostaglandin E2 Induces Potent T Helper 1–Inducing Dendritic Cells

Although the combination of OK432 and PGE_2 is beneficial for the induction of mature DCs with potent migration ability, some investigators have reported that PGE_2 reduces the T_H1 priming ability of MoDCs.[21] To examine such an effect of PGE_2 on DCs matured with OK432, we quantified IFNγ production from $CD4^+$ T cells primed with various DCs. DCs stimulated with OK432 alone (smDC-o and qmDC-o) significantly enhanced IFNγ secretion from $CD4^+$ T cells compared with immature DCs (siDCs and qiDCs), respectively (Fig. 4A). In addition, qmDC-o had a similar level of T_H1-inducing ability to that of smDC-o. The addition of PGE_2 tended to reduce the ability of these DCs to prime T cells to secrete IFNγ (smDC-op and qmDC-op), however (see Fig. 4A). Because qmDC-op demonstrated better migratory ability than smDC-op as mentioned in the previous section, we modified the qmDC-op generation protocol accordingly to alleviate T_H1 suppression by PGE_2. First, we titrated down the concentration of PGE_2. Next, we added T_H1-inducing reagent, IFNα, to the culture. In our preliminary experiment, IFNα enhanced the T_H1-stimulating ability of DCs in a dose-dependent manner over a range of 100 to 500 IU/mL and reached a plateau at 500 IU/mL (data not shown). Thus, we used 500 IU/mL IFNα in the following experiments to obtain a potent T_H1 response. We generated qmDCs with OK432 and different concentrations of PGE_2 (0–1000 ng/mL) in the presence or absence of IFNα. In the absence of IFNα, PGE_2 reduced the ability of qmDCs with OK432 to prime T_H1 in a dose-dependent manner (see Fig. 4B). The addition of IFNα enhanced the ability of priming T cells to produce IFNγ even in the presence of PGE_2, however, most significantly with a lower concentration of PGE_2 (50–100 ng/mL; see Fig. 4B).

As mentioned previously, the combination of 0.1 KE/mL OK432, 50 ng/mL PGE_2, and 500 IU/mL IFNα produced qmDCs with potent abilities of migration and T_H1 induction. We designated this combination of reagents as "OPA" (OK432, low-dose PGE_2, and IFNα) and used it as a maturation stimulus for quickly induced DCs in the following experiments. The qmDCs with OPA (OPA-DCs) expressed CD40, CD80, CD83, and CD86 comparable with qmDC-o and had potent migrating ability comparable with qmDC-op (data not shown).

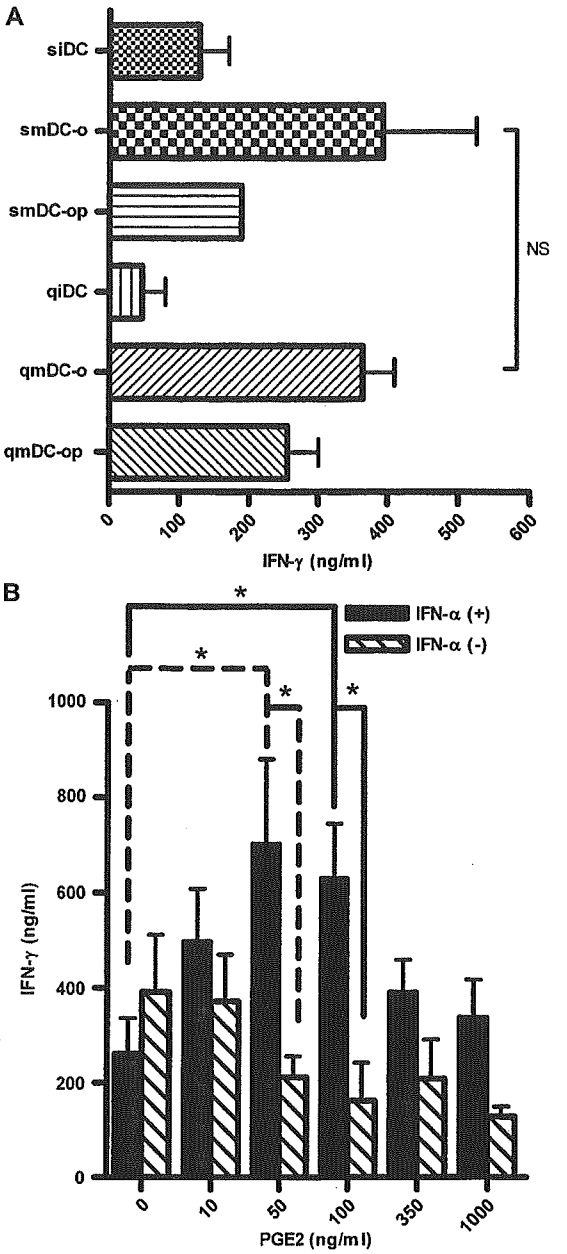


FIGURE 4. Addition of IFNα and reduction of PGE_2 alleviate the T_H1 suppressive effect of PGE_2 on qmDCs. Allogeneic naive $CD4^+$ T cells were cultured with various DCs for 7 days. Subsequently, the cells were harvested and stimulated with PMA and ionomycin for 24 hours. The amount of IFNγ in each supernatant was measured by ELISA. A, Various DCs were generated, as shown in Fig. 1, with 0.1 KE/mL OK432 or 350 ng/mL PGE_2. The results are expressed as the mean ± SEM of 6 experiments. B, qmDCs with 0.1 KE/mL OK432 and a different concentration of PGE_2 were generated in the presence (shaded bars) or absence (striped bars) of 500 IU/mL IFNα. The results are expressed as the mean ± SEM of 6 experiments ($^*P < 0.05$).

Quick Induction or the Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Stimuli Enhanced Yield of Monocyte-Derived Dendritic Cells

The yield of DCs is important for the potential clinical application of DC vaccine, because the number of DCs administered is one of the critical determinants for successful immunization. Thus, we compared the yield of various DCs (Fig. 5). With or without maturation stimuli, the quick induction method gave rise to the DCs with better yield compared with the conventional method. Furthermore, OPA-DCs gained

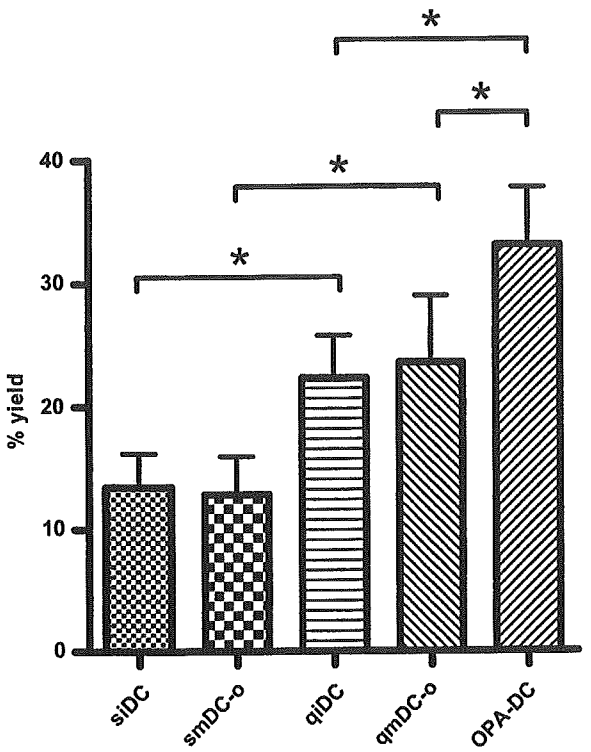


FIGURE 5. The quick induction and maturation with OPA enhanced the yield of MoDCs. Various DCs were generated with serum-free media, as described in Fig. 1. The yields of DCs were determined, as described in the Material and Methods section. The results are expressed as the mean ± SEM of 7 experiments (*$P < 0.05$).

higher yield than the other quickly induced DCs (qiDC and qmDC-o).

Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Is Comparable to the Monocyte-Conditioned Medium Mimic in Ability to Promote Migration and T Helper 1 Induction of Quickly Induced Mature Dendritic Cells

The combination of TNFα, IL-1β, IL-6, and PGE_2 is well known as an MCM-mimic cytokine cocktail, which many investigators have reported is effective for maturation of DCs.[2] Thus, we compared OPA (0.1 KE/mL OK432, 50 ng/mL PGE_2, and 500 IU/mL IFNα) with the MCM-mimic (10 ng/mL TNFα, 10 ng/mL IL-1β, 10 ng/mL IL-6, and 350 ng/mL PGE_2) with respect to their ability to improve migration and T_H1 induction of qiDCs. Interestingly, qmDCs with OPA (OPA-DCs) had a more potent migration ability than qmDCs with MCM-mimic (MCMm-DCs) (Fig. 6A). In addition, OPA-DCs primed $CD4^+$ T cells to produce T_H1 cytokine (IFNγ) more significantly than MCMm-DCs (see Fig. 6B), whereas their ability to stimulate $CD4^+$ T cells to produce IL-10 did not differ.

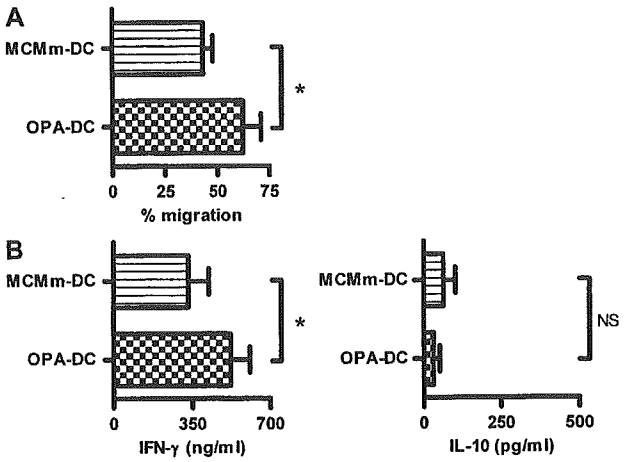


FIGURE 6. OPA has more potent ability than the MCM-mimic in stimulating DCs for migration and T_H1 induction. qiDCs were matured with OPA or the MCM-mimic for the last 24 hours of generation, as shown in Fig. 1. A, Migratory potential of these DCs toward CCL21 was analyzed, as described in the Materials and Methods section. B, Allogeneic naive $CD4^+$ T cells were cultured with each DC for 7 days. Subsequently, the cells were harvested and stimulated with PMA and ionomycin for 24 hours. The amounts of IFNγ and IL-10 in each supernatant were measured by ELISA. The results are expressed as the mean ± SEM of 10 experiments (*$P < 0.05$).

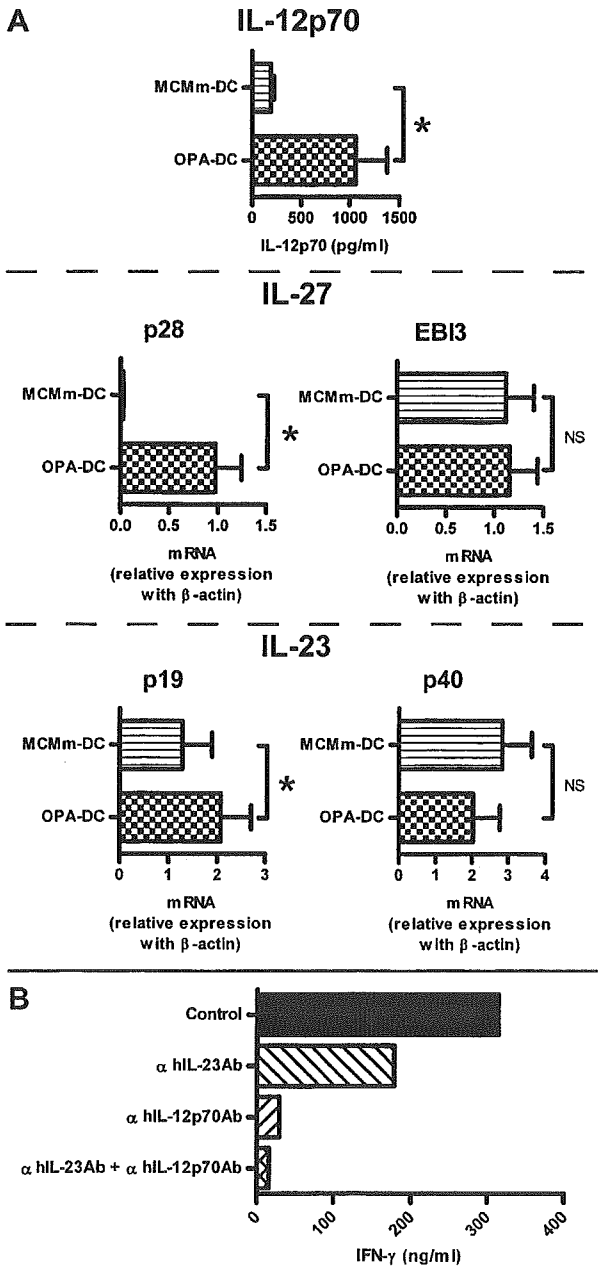
Quickly Induced Mature Dendritic Cells With Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Produce More Interleukin-12–Related Cytokines Than That With the Monocyte-Cultured Medium-Mimic

The cytokine-producing profile of DCs is one of the most important factors determining the helper T-cell responses. DC-derived IL-12 family members (IL12p70, IL-23, or IL-27) play a major role in orchestrating T_H1 responses.[22] OPA promoted DCs to secrete more IL-12p70 and to express more transcripts of p28 (a subunit of IL-27) and p19 (a subunit of IL-23) than the MCM-mimic (Fig. 7A). Neutralization of IL-12p70 in the coculture of DCs and $CD4^+$ T cells significantly reduced the T_H1-inducing ability of OPA-DCs (see Fig. 7B). The addition of anti-IL-23 antibody to the culture reduced DC-primed T_H1 induction, but much less than the additional of IL-12p70. Although the roles of IL-27 in the T-cell response are yet to be determined, these results indicate that IL-12p70 and IL-23 are primarily involved in OPA-DC–induced T_H1 polarization.

Quickly Induced Mature Dendritic Cells With Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Show Potent Natural Killer and Cytotoxic T-Lymphocyte–Inducing Ability

Many investigators have reported that NK cells and CTLs are major effector cells in the antitumor immune response. According to the results described previously, we compared OPA-DCs with MCMm-DCs for induction of NK cells and CTLs. We found that OPA-DCs activated NK cells

more significantly than MCMm-DCs (Fig. 8A). In addition, OPA-DCs primed CTLs to gain more potent lytic activity against CEA-peptide–pulsed target cells than MCMm-DCs (see Fig. 8B). In the presence of anti-human HLA-ABC antibody or anti-human CD8 antibody, the cells primed by OPA-DCs reduced their lytic activity to 25% to 30% of that without the antibody treatments, showing that they are HLA class I–restricted and CD8-positive cells. In addition, OPA-DC–primed cells showed limited lytic activity against unpulsed cells or K562 (data not shown). Thus, OPA-DCs could induce conventional CEA-peptide–specific CTLs with potent lytic capacities.



Quickly Induced Mature Dendritic Cells With Combination OK432, Low-Dose Prostaglandin E_2, and Interferon-α Obtained From Cancer Patients Have Comparable Activity to Those From Healthy Donors in Migration and T Helper 1 Stimulation

In advanced cancer patients, some functional disorders of DCs have been reported. To verify the feasibility of OPA-DCs as a therapeutic tool, we compared the abilities of OPA-DCs generated from cancer patients with those from healthy subjects. The profiles of the patients with untreated and advanced colonic or gastric cancer are shown in Figure 9A. The OPA enhanced CD40, CD80, CD83, CD86, and CCR7 expression on qiDCs of cancer patients as well as healthy donors (data not shown). In addition, OPA-DCs generated from cancer patients possessed comparable abilities to those from healthy volunteers in terms of migration and T_H1 induction (see Fig. 9B).

DISCUSSION

In clinical trials of DC vaccine against cancers, most have been carried out with MoDCs loaded with tumor lysate or a combination of peptides.[23] Unfortunately, the outcomes of these trials were not always satisfactory. Such limited responses of DC vaccine may be partly attributable to the immaturity of the DCs used. Because it has been demonstrated that mature DCs can induce more effective CTLs against cancers than immature DCs, several maturation stimuli for DCs have been examined to generate potent DCs in clinical settings. The protocols of DC generation still need to be improved to establish functionally mature DCs capable of enhancing in vivo anticancer immune responses, however.

Conventionally, MoDCs are generated in 5 to 7 days with subsequent maturation in 1 to 2 days, thus requiring a total of 7 to 9 days. Several drawbacks have been raised for this method with respect to clinical application. One is that the

FIGURE 7. qmDCs with OPA express more T_H1-inducing IL-12 family cytokines than those with the MCM-mimic. A, OPA-DCs and MCMm-DCs were stimulated by IFNγ and CD40L/L cells for 24 hours. The amount of IL-12p70 in each supernatant was measured by ELISA. The amounts of mRNA of IL-23 and IL-27 subunits in OPA-DCs and MCMm-DCs were measured by means of real-time quantitative RT-PCR. p19 and p40 are subunits of IL-23. p28 and EBI3 are subunits of IL-27. The mRNA expression of each subunit was standardized by β-actin mRNA expression as an internal standard. The results are expressed as the mean ± SEM of 6 experiments ($^*P < 0.05$). B, Allogeneic naive $CD4^+$ T cells were cultured with OPA-DCs for 7 days in the presence or absence of 25 ng/mL anti-human IL-12p70 antibody (αhIL-12Ab) and 20 ng/mL anti-human IL-23 antibody (αhIL-23Ab). As a control, isotype mouse IgG was used as a substitute for antibodies. Subsequently, the cells were harvested and stimulated with PMA and ionomycin for 24 hours. The amount of IFNγ in each supernatant was measured by ELISA. The representative results of 6 experiments are shown.

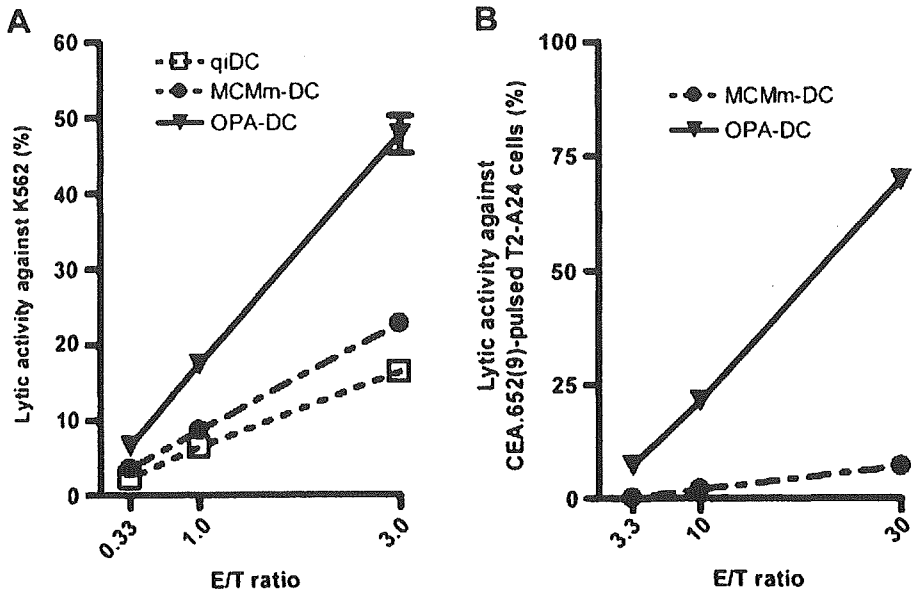


FIGURE 8. qmDCs with OPA significantly activate autologous NK cells and CEA-peptide–specific CTLs. A, Various qiDCs (qiDCs, MCMm-DCs, and OPA-DCs) were generated, as described in Fig. 1. After being stimulated, NK cells were cultured with K562 cells labeled with $Na_2{}^{51}CrO_4$ for 4 hours at 37°C. Supernatants were then harvested, and radioactivity was counted. The representative results of 5 experiments are shown. B, $CD14^-$, $CD56^-$, and $CD19^-$ cells were cultured with CEA 652(9)-loaded various DCs (MCMm-DCs and OPA-DCs) for 28 days with repetitive stimulation of IL-2 and IL-7, as described in the Materials and Methods section. They were then cocultured with CEA 652(9)-pulsed T2-A24 cells for 4 hours at 37°C. The supernatants were harvested, and radioactivity was counted. The representative results of 8 experiments are shown.

average yield of MoDCs cultured with media containing FCS or human serum by this protocol was 15% to 30%,[24,25] which limits the administration of sufficient numbers of DCs. Wong et al[26] reported that the yield of day 5 MoDCs was better than that of day 7, suggesting that a shorter culture period gives rise to larger numbers of DCs. In addition, quicker generation of DCs is beneficial for the maintenance of cell quality, because the apoptosis rate of DCs is reported to increase from 10% to 25% over 1 week of DC culture.[27] Thus, MoDCs should be generated in short-time culture if the cells are phenotypically and functionally comparable with mature DCs. Recently, Dauer et al[15] reported that mature DCs are inducible in only 2 days using a cytokine cocktail of TNFα, IL-1β, IL-6, and PGE_2, which is well known as an MCM-mimic.[2] In support of this report, we demonstrated in this study that OK432 can be used as a substitute for the MCM-mimic to generate mature DCs in 3 days. The average yield of DCs generated by this short-term protocol was more than 30%, even with serum-free media.

The migratory capability of DCs toward DLs is one of the essential factors dictating the successful induction of anti-tumor immune responses.[11,23] In this process, the interactions of chemokines expressed in secondary lymphoid tissue (CCL21) and its receptor (CCR7) on DCs play a major role. Recently, PGE_2 combined with TNFα or IL-1β was found to promote CCR7 expression of mature MoDCs.[20] In the present study, we have shown that a combination of PGE_2 and OK432 significantly enhanced CCR7 expression and migration of DCs to CCL21. Interestingly, qmDC-op demonstrated better CCR7 expression and migrating ability than smDC-op. Although the molecular mechanisms for these processes remain to be determined, one of the reasons may be that qmDC-op maintained better biologic capacity than smDC-op, because the viability of qmDCs was better as a result of the short culture period (data not shown).

Regardless of the positive impact of PGE_2 on DC migration, many investigators have reported that PGE_2 inhibits the T_H1-stimulating activity of MoDCs.[21,28] In the present study, PGE_2 also suppressed the T_H1-stimulating activity of day 7 and day 3 OK432-primed DCs in a dose-dependent manner. To overcome such an inhibitory effect of PGE_2, we reduced the concentration of PGE_2 and added IFNα to generate day 3 mature DCs. Consequently, the addition of IFNα and low dose of PGE_2 to OK432 at the early phase of DC differentiation significantly enhanced the T_H1-inducing activity of MoDCs. Recently, several investigators have reported that IL-12 family members (IL-27, IL-12p70, and IL-23) play major roles in the differentiation of T_H1 cells.[22] Although the MCM-mimic has been widely used as a DC maturation stimulus, it is not always sufficient to endow DCs with the ability to produce biologically active IL-12.[6] In this study, OPA-DCs significantly secreted more IL-12p70 and induced more transcripts of p28 and p19 (subunits of IL-27 and IL-23, respectively) than MCMm-DCs. In addition, neutralization of IL-23 and IL-12p70 with antibodies reduced the T_H1-inducing ability of OPA-DCs. Therefore, enhanced IL-12p70 and IL-23 expression may play important roles in the potent T_H1 induction with OPA-DCs. Further examination is

A

Patient No.	Age	Gender	Primary lesion	Metastasis	CEA# (ng/ml)
1	67	male	colon	(-)	37.0
2	66	male	stomach	(+) p.c.*	413.0
3	61	male	stomach	(+) p.c.*	2.3
4	64	female	colon	(-)	6.4
5	68	female	stomach	(-)	1.1
6	68	female	colon	(-)	12.7
7	62	male	colon	(-)	2.7
mean	65.1				67.9

* p.c. = peritonitis carcinomatosa

#The normal range of CEA is less than 5 ng/ml.

B

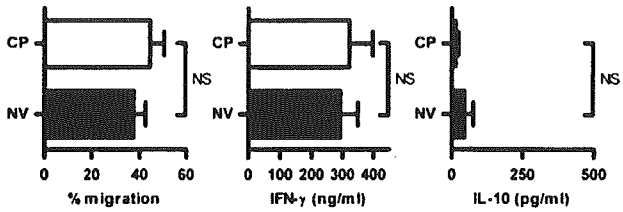


FIGURE 9. OPA can quickly generate mature DCs with potent abilities of migration and T_H1 induction even from monocytes of cancer patients. *A*, Clinical backgrounds of the advanced cancer patients are presented. None of the patients was infected with hepatitis B virus (HBV) or hepatitis C virus (HCV). *B*, OPA-DCs were generated from cancer patients (CP) and normal volunteers (NV). The normal volunteers were blood donors who were negative for human immunodeficiency virus (HIV), HBV, and HCV. The migratory potential of OPA-DCs derived from CP or NV was analyzed, as described in the Materials and Methods section. Conversely, $CD4^+$ T cells primed with OPA-DCs for 7 days were stimulated with PMA and ionomycin for 24 hours. The amount of IFNγ and IL-10 in each supernatant was measured by ELISA. The results are expressed as the mean ± SEM of 7 experiments.

needed to determine whether or not IL-27 is involved in the OPA-DC–induced T_H1 response.

It is still unknown why an optimum concentration of PGE_2 (50–100 ng/mL) exists in the combination of IFNα and OK432 to enhance the T_H1-inducing ability of qmDCs. In our unpublished data, IFNα significantly enhanced IL-12p70 and IL-27 expression in qmDCs but showed limited enhancement of IL-23 transcripts. Conversely, PGE_2 significantly increased IL-23 transcripts but reduced IL-12p70 and IL-27 expression in a dose-dependent manner (data not shown). Therefore, in the presence of OK432 and IFNα, it is conceivable that low-dose PGE_2 is required for qmDCs to maintain sufficient expression of IL-23 without decreasing IL-12p70 production for T_H1 polarization.

We found that OPA enhanced the ability of qmDCs to stimulate NK cells as well as to induce CEA-peptide–specific CTLs more than the MCM-mimic. A plausible reason for this is the profound ability of OPA-DCs to secrete IL-12p70 and induce T_H1, because it has been demonstrated that IL-12p70 and T_H1-derived IFNγ contribute to the activation of NK cells and CTLs, respectively.

Some investigators have reported that DCs obtained from cancer patients have functional disorders,[29] including impaired maturation in response to inflammatory stimuli. Such DC dysfunction may be related, at least in part, to unsatisfactory outcomes of DC vaccine trials against advanced cancers. We demonstrated in the present study that the OPA cocktail could induce phenotypically and functionally mature MoDCs even from advanced cancer patients, suggesting that OPA is a feasible immune adjuvant for a DC vaccine.

In summary, we successfully generated novel mature DCs with OPA. This type of DC possesses the characteristics of quick inducibility, potent migrating ability, and potent stimulating activity for T_H1, CTL, and NK cells, which are desirable for DC vaccines against cancers.

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Natural Killer Cells in Hepatitis C Virus Infection: From Innate Immunity to Adaptive Immunity

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Natural killer (NK) cells are specialized lymphocytes that provide a first line of defense through their ability to kill pathogen-infected cells and transformed cells. The function of NK cells is regulated by a fine balance of inhibitory and activating signals, which are mediated by a diverse array of cell-surface receptors. We recently found that expression of the inhibitory receptor CD94/NKG2A is up-regulated on NK cells in patients with chronic hepatitis C. HLA-E, a ligand for NKG2A, was expressed in all human hepatoma cell lines tested as well as in nontransformed hepatocytes, but not in K562 cells, a classic NK-sensitive target. NK cells isolated from patients with chronic hepatitis C (HCV-NK) were less capable of killing hepatoma cells and of producing interferon-γ in response to hepatoma cells than those from healthy donors, whereas there was no significant difference in NK responsiveness toward K562 cells. Of note is the finding that maturation and activation of monocyte-derived dendritic cells were negatively modulated in the presence of HCV-NK and hepatoma cells, which were restored by the addition of anti-NKG2A antibody during the coculture of HCV-NK and hepatoma cells. Research revealed that dendritic cells recognize danger signals from microorganisms by monitoring pathogen-associated molecular patterns via Toll-like receptors. Our findings have shed light on NK receptors as an important interface that transmits danger signals from abnormal cells to immune systems. Aberrant expression of CD94/NKG2A should have negative impact on innate resistance and subsequent adaptive immunity toward HCV-infected or transformed cells in chronic hepatitis C.

Immune responses are generally divided into 2 types, innate immunity and adaptive immunity. The cellular components of the innate immunity include natural killer (NK) cells and natural killer T cells, whereas those of the adaptive immunity consist of T cells and B cells. A dendritic cell (DC) is a most potent antigen-presenting cell that can activate naïve T cells, serving as a sentinel between innate immunity and adaptive immunity. Most research on HCV immunobiology has focused on the adaptive immunity.[1] This includes low frequencies and a reduced capacity of cytotoxic T cells, low frequency of $CD4^+$ T cells targeting a limited number of epitopes, and T-helper 2 (Th2) predominance. However, little is known about the innate immunity.

NK cells are specialized lymphocytes that provide a first line of defense through their ability to kill pathogen-infected cells and transformed cells. The function of NK cells is regulated by a fine balance of inhibitory and activating signals, which are mediated by a diverse array of cell-surface receptors.[2] Inhibitory receptors include a variety of killer cell immunoglobulin-like receptors (KIRs) and C-type-lectin receptors such as CD94/NKG2A. These receptors bind self–major histocompatibility complex (MHC) class I molecules, which are constitutively expressed in normal cells. If the expression of self–class I molecules decreases in a target cell, it will result in decreased inhibitory signals and activation of NK cells. Thus, NK inhibitory receptors serve as a recognition system for the "missing-self." On the other hand, NKG2D, a typical example of NK activating receptors, binds non-classic MHC class I molecules that are not expressed in normal cells but selectively induced on epithelial cells on exposure to a variety of stresses such as infection and transformation. Therefore, it serves as a recognition system for the "altered-self."

The importance of NK cells in HCV infection is increasingly being recognized. First, there was the report of a certain percentage of HCV-infected chimpanzees displaying spontaneous clearance of infection without developing adaptive immunity.[3] Also, NK cells were found to inhibit replication of HCV in replicon-containing hepatic cells via an interferon-γ (IFN-γ)–dependent mechanism.[4] The impact of NK cells on the resolution of

Abbreviations used in this paper: DC, dendritic cell; HCV-NK, natural killer cells from patients with chronic hepatitis C; IFN, interferon; KIR, killer cell immunoglobulin-like receptor; MHC, major histocompatibility complex; MoDC, monocyte-derived dendritic cell; NK, natural killer; N-NK, natural killer cells from healthy donors; Th, T-helper.

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HCV infection was made evident by a recent article[5] that presented epidemiologic evidence for an association between specific KIR/HLA compound genotypes and the outcome of HCV infection. Subjects who were homozygous for KIR2DL3 and group 1 HLA-C alleles were more likely to resolve HCV infection than patients with any other KIR/HLA compound genotype. Of interest is the finding that the association between KIR2DL3/HLA-C1 and HCV clearance was statistically significant only for those subjects who had been infected via use of injection drugs or accidentally being stuck with a needle and not for those who had been transfused with blood products. These results suggest that NK cells might have a critical role in the clearance of HCV infection induced by low-dose exposure.

With respect to NK activity in HCV infection, in vitro experiments have revealed that HCV glycoprotein E2 is capable of binding to CD81 and thus inhibiting NK cell activity.[6,7] However, there is some discrepancy in NK cell activity in chronic HCV infection.[8] Some have reported reduced NK activity in chronic HCV infection, whereas others have denied it.

Natural Killer Receptor Expression and Effector Cell Functions

We initially examined NK cell responsiveness in chronic hepatitis C.[9] NK cells isolated from peripheral blood were cocultured for 24 hours with either Hep3B hepatoma cells or K562 cells, a classic NK-sensitive target, and subjected to flow cytometric analysis for IFN-γ production. NK cells from patients with chronic hepatitis C (HCV-NK), on exposure to K562 cells, produced IFN-γ at levels similar to those of NK cells isolated from healthy donors (N-NK). In contrast, HCV-NK cells did not produce IFN-γ in response to Hep3B on comparison with N-NK cells.

On the basis of these findings, we hypothesized that the NK cell receptor expression profile might differ between HCV patients and normal donors. We examined the levels of various NK cell receptors on $CD56^+$ cells in the peripheral blood by flow cytometry. There was no significant difference in the expression of all KIRs tested between HCV patients and healthy donors, whereas the expression of CD94 and NKG2A was significantly higher in HCV patients than in normal volunteers. There was no difference in the expression of the NKG2D activating receptor.

NKG2A and CD94 compose a heterodimeric receptor. This receptor binds highly conserved HLA-E, whose expression requires peptides derived from signal sequences of HLA-A, HLA-B, or HLA-C. If the expression

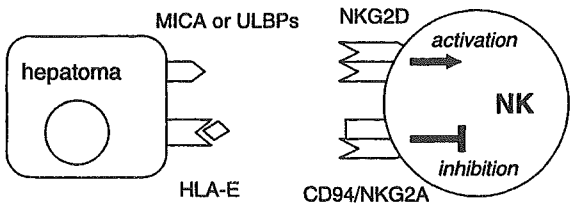

Figure 1. Regulation of NK cell functions by hepatoma cells. *MICA*, MHC class I–related chain A; *ULBPs*, UL16 binding proteins.

of these MHC antigens decreases in a cell, it will also result in decreased expression of HLA-E. NK cells monitor the overall expression of MHC class I antigens on target cells by simply monitoring the expression of the HLA-E antigen via NKG2A/CD94 receptors. This is in sharp contrast to the variety of KIRs that bind highly polymorphic HLA-B and HLA-C in an allele-specific manner.

Now the question arises of whether HLA-E is expressed in hepatic cells. HLA-E was expressed on all hepatoma cell lines tested and nontransformed hepatocytes, but not on K562 cells. Next, we performed the chromium release assay for NK cytolysis against Hep3B cells or K562 cells. HCV-NK cells killed K562 cells at levels similar to those of N-NK cells. In contrast, HCV-NK cells killed Hep3B cells less effectively than N-NK cells did. To examine the possible involvement of the NKG2A expression in the decreased NK cytolytic ability against Hep3B cells found in HCV patients, we added anti-NKG2A antibody during the coculture of effector cells and target cells. The addition of anti-NKG2A antibody did not affect the killing activity against K562 cells. However, anti-NKG2A did increase the killing activity against Hep3B cells. Importantly, the extent of the up-regulation was greater for HCV-NK than for N-NK.

We previously reported that hepatoma cells expressed NKG2D ligands such as MHC class I–related chain A and UL16 binding proteins.[10] In the presence of hepatoma cells, NK cell activity is regulated by a fine balance of the activating signal via NKG2D and inhibitory signals via CD94/NKG2A (Figure 1). In patients with chronic hepatitis C, CD94/NKG2A expression is up-regulated on NK cells, which causes decreased NK cell responsiveness toward HLA-E–expressing hepatic cells.

Impact of Natural Killer Cells on Adaptive Immunity

Originally, the NK cell was thought to be just an effector cell for killing unwanted cells. However, recent research has been shedding light on its role as a regulator for the subsequent adaptive immune responses. For ex-

ample, there have been reports that NK cells activated DCs via a tumor necrosis factor–α–dependent mechanism,[11] and that NK cells down-regulated an immune response by killing immature DCs.[12] Immature DCs distributed into the peripheral organs such as the liver undergo maturation to initiate immune responses under pathologic conditions. It is well-known that the liver contains a high percentage of NK cells. We sought to examine the possibility that interaction of NK cells and hepatic cells might affect the maturation status of DCs. To this end, we cultured NK cells and monocyte-derived DCs (MoDCs) at a ratio of 1 to 1 with Hep3B cells. Two days later, flow cytometric analysis was performed to analyze the maturation markers such as CD40, CD86, HLA-DR, and CD83 on DCs. MoDCs showed immature phenotypes. DCs continued to show immature phenotypes when cocultured only with NK cells. In contrast, DCs clearly matured when cocultured with NK cells and Hep3B cells. However, maturation of DCs was suppressed, when HCV-NK cells were used instead of N-NK cells.

DC maturation induced by NK cells and hepatoma cells does not require cell-to-cell contact because insertion of the trans-well membrane between DC and NK/Hep3B did not affect the results. Thus, to simplify the experimental system, we first cocultured Hep3B cells and NK cells isolated from either HCV-infected patients or healthy donors for 1 day. MoDCs isolated from allogeneic healthy donors were stimulated by NK and Hep3B conditional medium. In agreement with the triple culture data, N-NK and Hep3B conditional medium induced maturation of DCs, whereas HCV-NK and Hep3B conditional medium failed to induce DC maturation. In contrast, N-NK and K562 conditional medium as well as HCV-NK and K562 conditional medium did induce maturation of DCs, suggesting that HLA-E–NKG2A interaction might be involved in this phenomenon.

To examine whether maturation of DC is functionally relevant, we cocultured graded numbers of DCs with allogeneic T cells and examined the proliferative responses after 5 days. MoDCs did not activate allogeneic T cells. DCs stimulated by conditional medium of NK cells isolated from either HCV-infected patients or healthy donors did not activate allogeneic T cells. In contrast, DCs stimulated by NK and K562 conditional medium were capable of activating T cells. DCs stimulated by N-NK and Hep3B conditional medium could also activate allogeneic T cells, but DCs stimulated by HCV-NK and Hep3B conditional medium did not function efficiently. To examine whether HLA-E–NKG2A interaction is involved in down-regulation of DC maturation and activation, we added anti-NKG2A antibody during the coculture of NK cells and Hep3B cells. This caused the up-regulation of the allostimulatory capacity of DCs stimulated by NK-Hep3B conditional medium. Importantly, the up-regulation was prominent for HCV patients, and the allostimulatory capacity was almost completely restored in comparison with healthy donors.

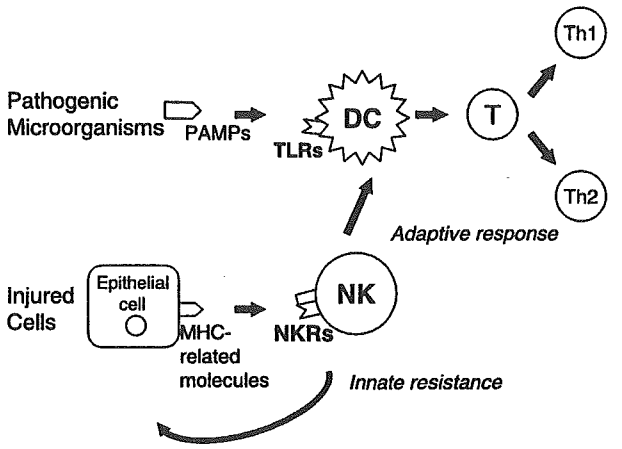

Figure 2. Transmission of danger signals of pathogens and tumors to the immune systems. *TLRs*, Toll-like receptors; *NKRs*, NK cell receptors; *PAMPs*, pathogen-associated molecular patterns.

DC is the most potent antigen-presenting cell, which activates naïve T cells and primes Th1 and Th2 responses. Toll-like receptors expressed on DCs recognize pathogen-associated molecular patterns to mature and activate DCs. Thus, Toll-like receptor is an important interface that can transmit danger signals of pathogens to the immune system. In contrast, cellular abnormalities such as altered-self or missing-self can be recognized by various NK receptors expressed on NK cells. NK cells activated by transformed or injured cells not only directly kill these cells but produce a variety of cytokines to activate the subsequent adaptive immune responses. Thus, NK receptors serve as an important interface that transmits cellular abnormalities to immune systems (Figure 2). In HCV infection, NK cells overexpressed the NK inhibitory receptor CD94/NKG2A, leading to down-regulation of innate resistance to HLA-E expressing hepatoma cells as well as down-modulation of T-cell activation. Altered expression of CD94/NKG2A might be involved in impaired innate recognition as well as the subsequent adaptive immune response to pathogens and tumors in chronic HCV infection.

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