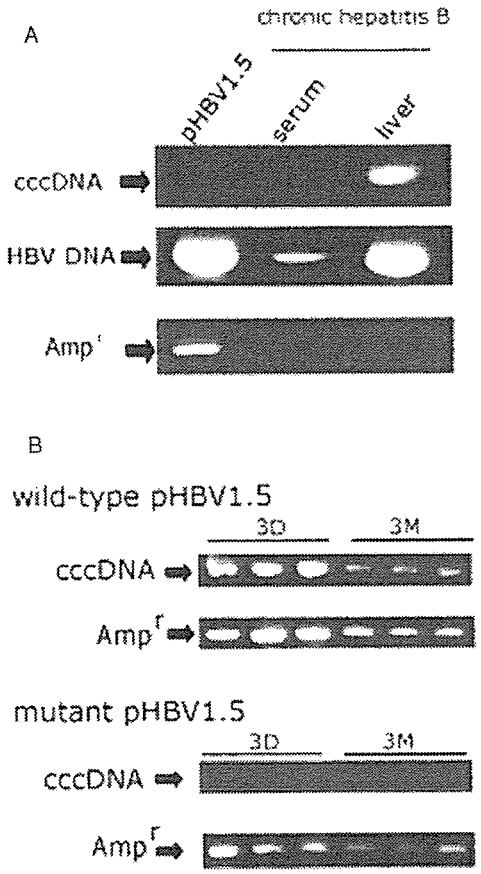

Fig. 5. Detection of HBV cccDNA and ampicilin resistance gene by PCR. (A) Specificity of HBV cccDNA detection by PCR. DNA were isolated from the following samples and amplified for the detection of cccDNA, ampicilin resistance gene, and HBV DNA sequence by corresponding PCRs. pHBV1.5, sample containing 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

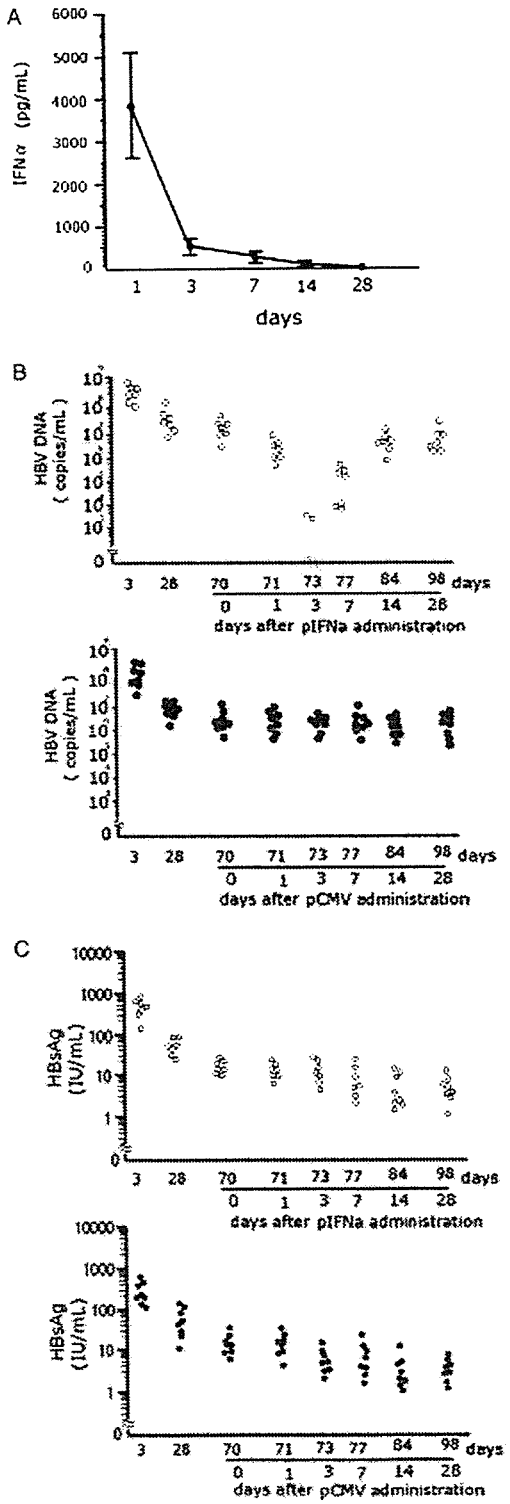

Fig. 6. Transient suppression of HBV production by IFNα gene therapy. (A) Serial determination of serum IFNα levels of nude mice after injection of pCMV-IFNa1. Horizontal bars indicate SD. (B and C) Nude mice were transfected with pHBV1.5 and, at 70 days later, transfected with either pCMV-IFNa1 (open 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

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan.

References

[1] Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med 2004;350:1118–1129.
[2] Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. Lancet 2003;362:2089–2094.
[3] Feitelson MA, Larkin JD. New animal models of hepatitis B and C. ILAR J 2001;42:127–138.
[4] Araki K, Miyazaki J, Hino O, Tomita N, Chisaka O, Matsubara K, et al. Expression and replication of hepatitis B virus genome in transgenic mice. Proc Natl Acad Sci USA 1989;86:207–211.
[5] Guidotti LG, Matzke B, Schaller H, Chisari FV. High level hepatitis B virus replication in transgenic mice. J Virol 1995;69:6158–6169.
[6] Takahashi H, Fujimoto J, Hanada S, Isselbacher KJ. Acute hepatitis in rats expressing human hepatitis B virus transgenes. Proc Natl Acad Sci USA 1995;92:1470–1474.
[7] Suzuki T, Takehara T, Ohkawa K, Ishida H, Jinushi M, Miyagi T, et al. Intravenous injection of naked plasmid DNA encoding hepatitis B virus (HBV) produces HBV and induces humoral immune response in mice. Biochem Biophys Res Commun 2003;300:784–788.
[8] Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. Proc Natl Acad Sci USA 2002;99:13825–13830.
[9] McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, et al. Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol 2003;21:639–644.
[10] Klein C, Bock CT, Wedemeyer H, Wustefeld T, Locarnini S, Dienes HP, et al. Inhibition of hepatitis B virus replication in vivo by nucleoside analogues and siRNA. Gastroenterology 2003;125:9–18.
[11] Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol Ther 2003;8:769–776.
[12] Harle P, Noisakran S, Carr DJ. The application of a plasmid DNA encoding IFN-alpha 1 postinfection enhances cumulative survival of herpes simplex virus type 2 vaginally infected mice. J Immunol 2001; 166:1803–1812.
[13] Liu F, Song YK, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 1999;6: 1258–1266.
[14] Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injection of naked plasmid DNA. Hum Gene Ther 1999;10:1735–1737.
[15] Kawanishi Y, Hayashi N, Katayama K, Ueda K, Takehara T, Miyoshi E, et al. Tumor necrosis factor-α and interleukin-γ inhibit synergistically viral replication in hepatitis B virus-replicating cells. J Med Virol 1995;47:272–277.
[16] Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantification of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;37:2899–2903.
[17] Jun-Bin S, Zhi C, Wei-Quin N, Jun F. A quantitative method to detect HBV cccDNA by chimeric primer and real-time polymerase chain reaction. J Virol Methods 2003;112:45–52.
[18] Kaplan PM, Ford EC, Purcell RH, Gerin JL. Demonstration of subpopulation of Dane particles. J Virol 1976;17:885–893.
[19] Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev 2000;64:51–68.
[20] Harweijer H, Zhang G, Subbotin VM, Budker V, Williams P, Wolff JA. Time course of gene expression after plasmid DNA gene transfer to the liver. J Gene Med 2001;3:280–291.
[21] Ueda K, Tsurimoto T, Nagahata T, Chisaka O, Matsubara K. An in vitro system for screening anti-hepatitis B virus drugs. Virology 1989;169:213–216.
[22] Feitelson MA, DeTolla LJ, Zhou XD. A chronic carrierlike state is established in nude mice injected with cloned hepatitis B virus DNA. J Virol 1988;62:1408–1415.
[23] Jiang J, Yamato E, Miyazaki J. Intravenous delivery of naked plasmid DNA for in vivo cytokine expression. Biochem Biophys Res Commun 2001;289:1088–1092.
[24] Yang J, Chen S, Huang L, Michalopoulos GK, Liu Y. Sustained expression of naked plasmid DNA encoding hepatocyte growth factor in mice promotes liver and overall body growth. Hepatology 2001;33: 848–859.
[25] Delaney IV WE, Isom HC. Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus. Hepatology 1998;28:1134–1146.
[26] Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–113.
[27] Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–1974.

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

Mitsuru Sakakibara, Tatsuya Kanto,*† Michiyo Inoue,*† Aki Kaimori,* Takayuki Yakushijin,* Hideki Miyatake,* Ichiyo Itose,* Masanori Miyazaki,* Noriyoshi Kuzushita,* Naoki Hiramatsu,* Tetsuo Takehara,* Akinori Kasahara,‡ and Norio Hayashi**

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.

Received for publication June 6, 2005; accepted August 11, 2005.

From the *Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan; †Department of Dendritic Cell Biology and Clinical Applications, Osaka University Graduate School of Medicine, Osaka, Japan; and ‡Department of General Medicine, Osaka University Graduate School of Medicine, Osaka, Japan.

Supported by a grant-in-aid for Research on Emerging and Re-Emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan; a grant-in-aid from the Ministry of Education, Science, and Culture of Japan; and the Organization for Pharmaceutical Safety and Research.

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

Reprints: Norio Hayashi, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan (e-mail: hayashin@moltx.med.osaka-u.ac.jp).

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

(*J Immunother* 2006;29:67–77)

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,

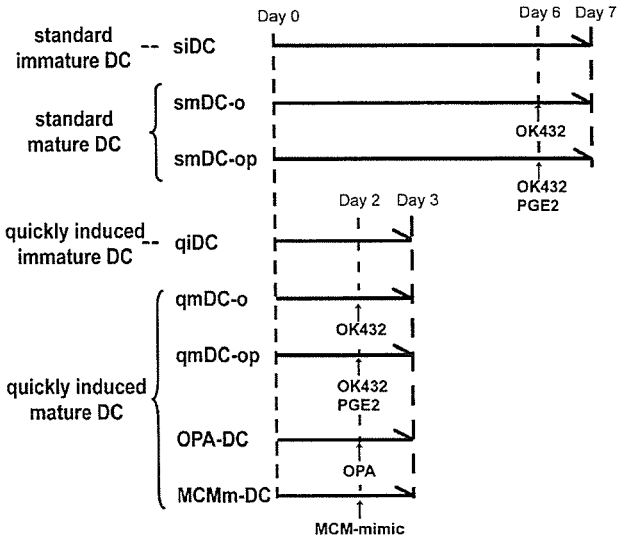

FIGURE 1. Preparation of DCs generated by means of various protocols. siDCs are generated with GM-CSF and IL-4 over 7 days. smDCs are siDCs stimulated with OK432 in the absence (smDC-o) or presence (smDC-op) of 350 ng/mL PGE_2 for the final 24 hours. qiDCs are generated from monocytes with IL-4 and GM-CSF 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

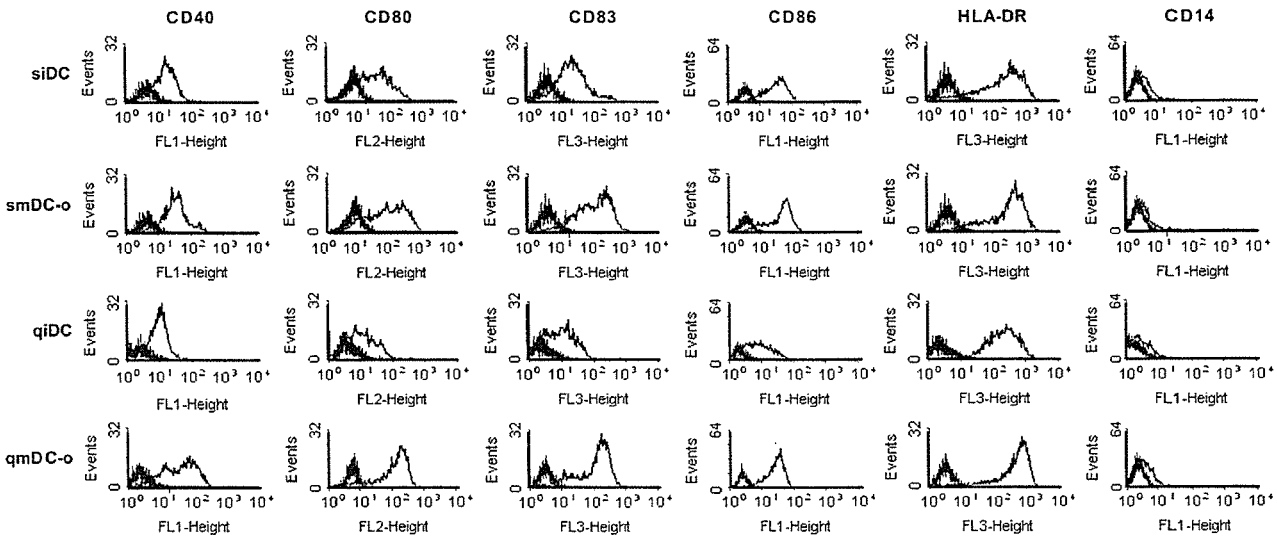

FIGURE 2. OK432 induces maturation on qiDCs and siDCs. Cells differentiated under distinct culture conditions were analyzed by flow cytometry for the expression of CD40, CD80, CD83, CD86, HLA-DR, and CD14 (thin lines with a shaded portion). Thick lines with an unshaded portion show the results of cells 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.

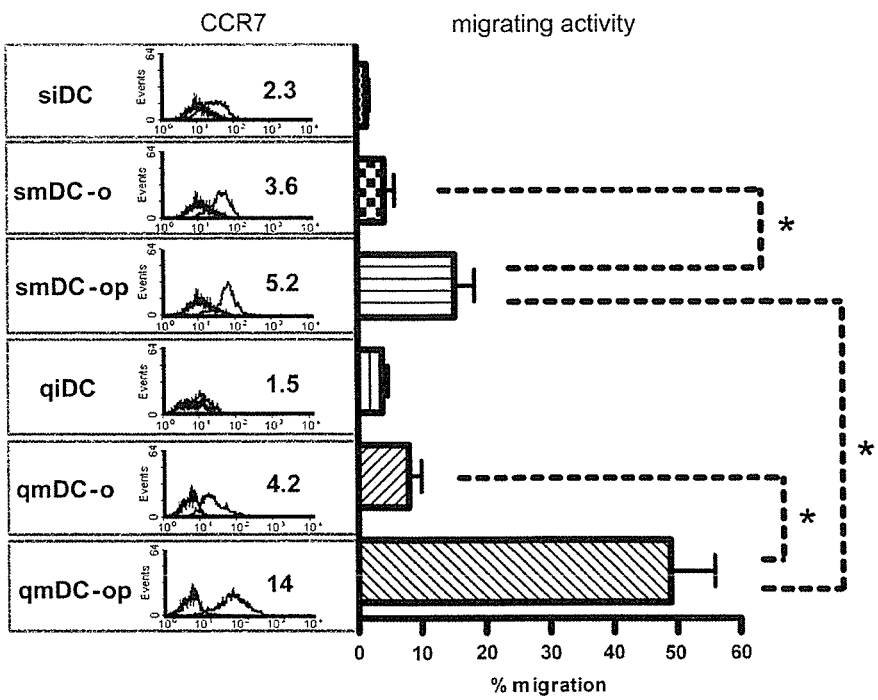

FIGURE 3. PGE_2 combined with OK432 enhances CCR7 expression and migration to CCL21 of qiDCs and siDCs. Various DCs were generated, as described in Fig. 1. They were analyzed by flow cytometry for the expression of CCR7. Thin lines with a shaded portion show CCR7 expression of various DCs. Thick lines 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).

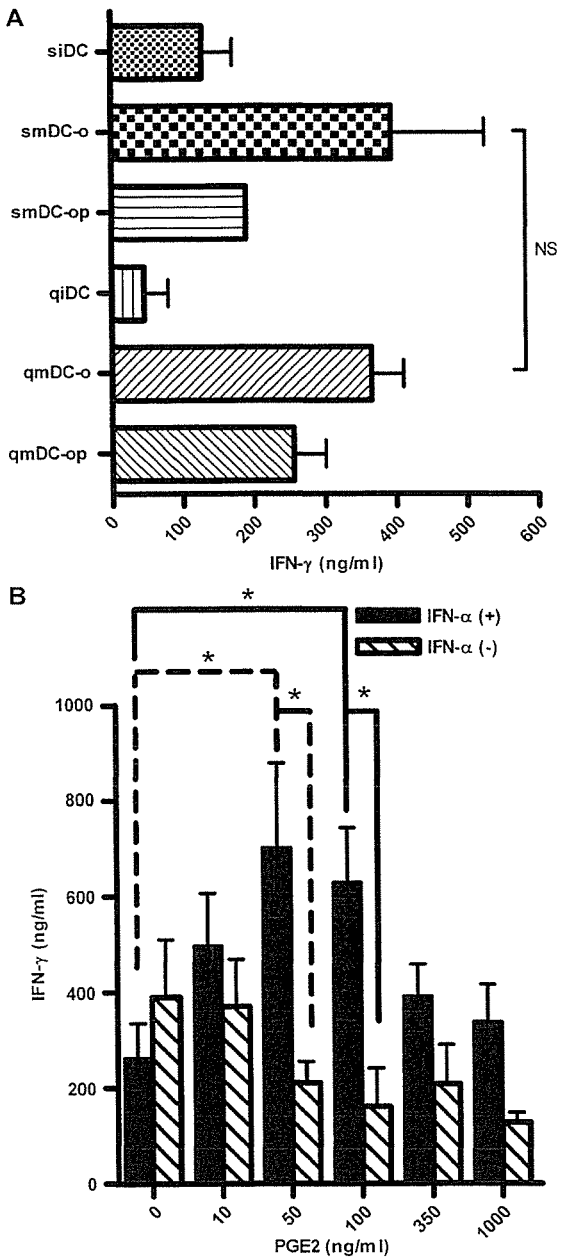

FIGURE 4. Addition of IFNα and reduction of PGE_2 alleviate the T_H1 suppressive effect of PGE_2 on qmDCs. Allogeneic naive $CD4^+$ T cells were cultured with various DCs for 7 days. Subsequently, the cells were harvested and stimulated with PMA and ionomycin for 24 hours. The amount of IFNγ in each 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

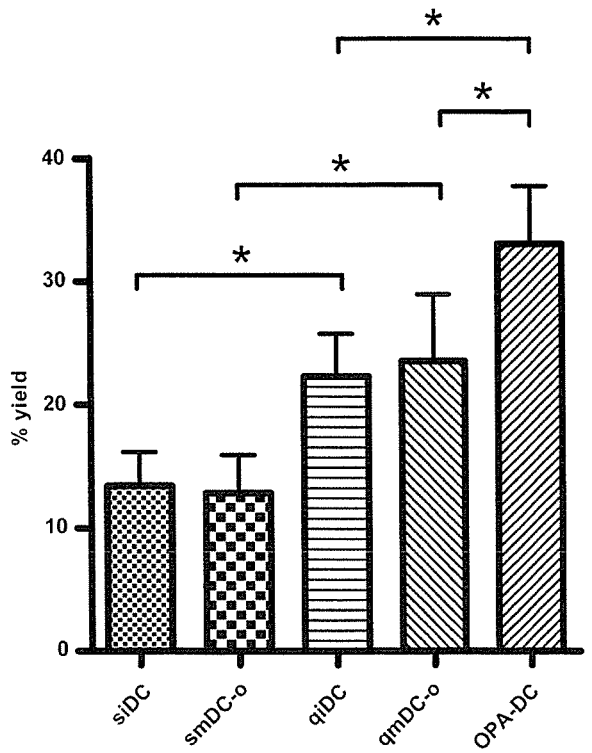

FIGURE 5. The quick induction and maturation with OPA enhanced the yield of MoDCs. Various DCs were generated with serum-free media, as described in Fig. 1. The yields of DCs were determined, as described in the Material and Methods section. The results are expressed as the mean ± SEM of 7 experiments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

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.

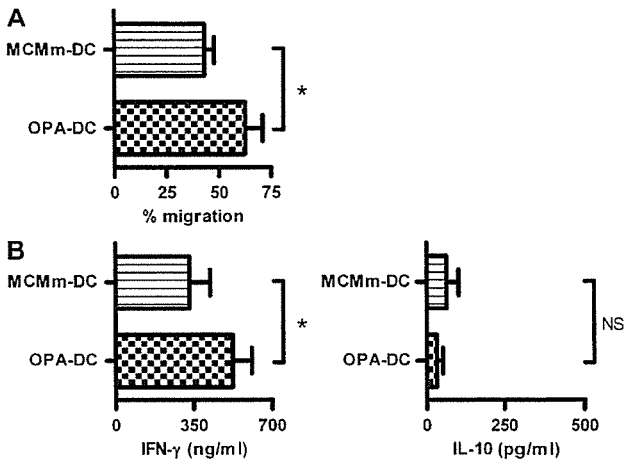

FIGURE 6. OPA has more potent ability than the MCM-mimic in stimulating DCs for migration and T_H1 induction. qiDCs were matured with OPA or the MCM-mimic for the last 24 hours of generation, as shown in Fig. 1. A, Migratory potential of these DCs toward CCL21 was analyzed, as described in the Materials 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.

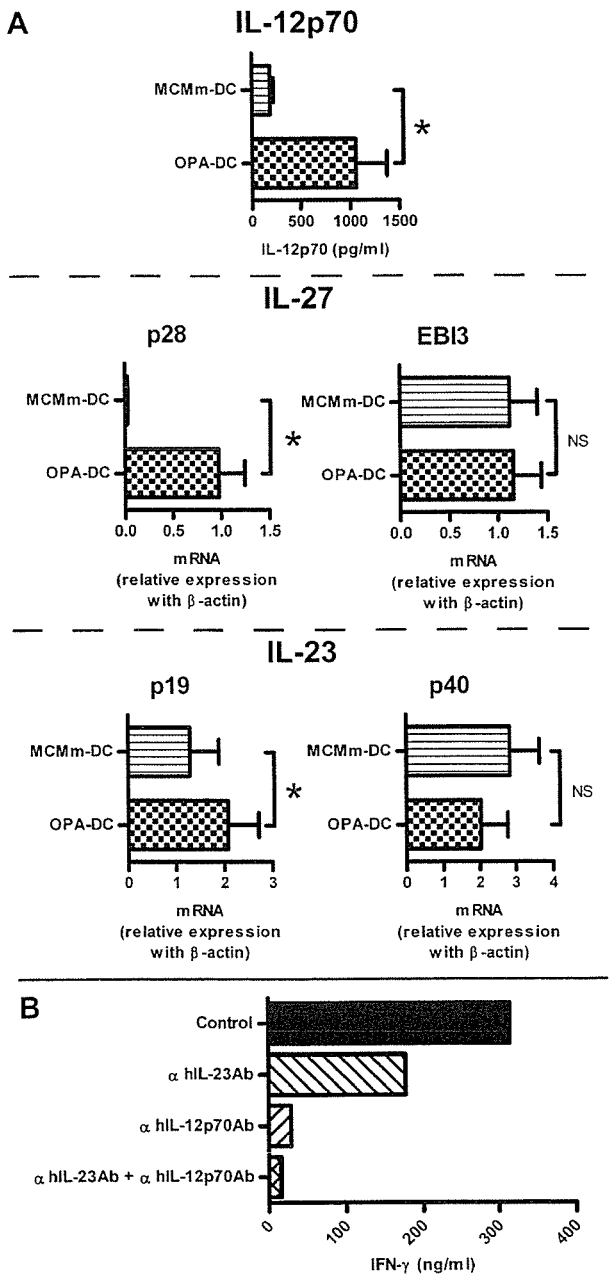

FIGURE 7. qmDCs with OPA express more T_H1-inducing IL-12 family cytokines than those with the MCM-mimic. A, OPA-DCs and MCMm-DCs were stimulated by IFNγ and CD40L/L cells for 24 hours. The amount of IL-12p70 in each supernatant was measured by ELISA. The amounts of mRNA of IL-23 and IL-27 subunits in 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.

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

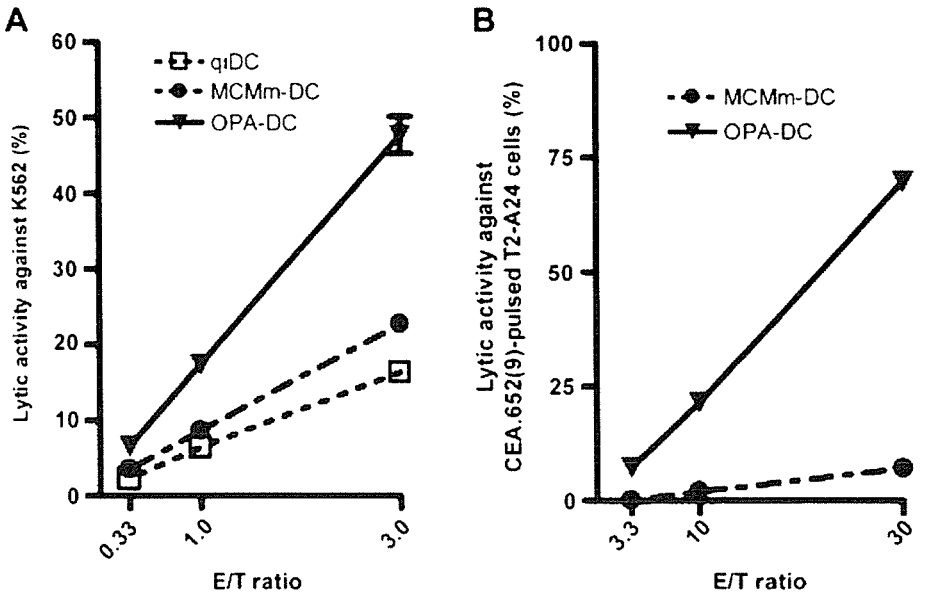

FIGURE 8. qmDCs with OPA significantly activate autologous NK cells and CEA-peptide–specific CTLs. A, Various qiDCs (qiDCs, MCMm-DCs, and OPA-DCs) were generated, as described in Fig. 1. After being stimulated, NK cells were cultured with K562 cells labeled with $Na_2{}^{51}CrO_4$ for 4 hours at 37°C. 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

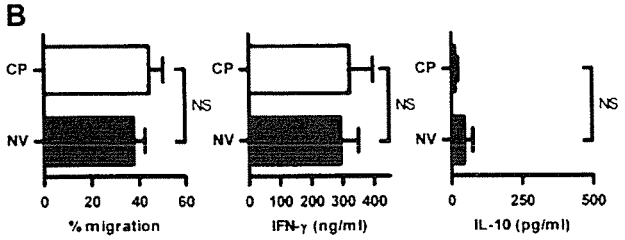

FIGURE 9. OPA can quickly generate mature DCs with potent abilities of migration and T_H1 induction even from monocytes of cancer patients. *A*, Clinical backgrounds of the advanced cancer patients are presented. None of the patients was infected with hepatitis B virus (HBV) or hepatitis C virus (HCV). *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.

ACKNOWLEDGMENTS

The authors thank Dr. Takashi Abe (Osaka Police Hospital) and Dr. Eiji Mita (Saiseikai Senri Hospital) for providing blood samples from cancer patients.

REFERENCES

1. Ridgway D. The first 1000 dendritic cell vaccines. *Cancer Invest.* 2003; 21:873–886.
2. Jonuleit H, Kuhn U, Muller G, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol.* 1997;27: 3135–3142.
3. de Vries IJ, Lesterhuis WJ, Scharenborg NM, et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. *Clin Cancer Res.* 2003;9:5091–5100.
4. Vilella R, Benitez D, Mila J, et al. Pilot study of treatment of biochemotherapy-refractory stage IV melanoma patients with autologous dendritic cells pulsed with a heterologous melanoma cell line lysate. *Cancer Immunol Immunother.* 2004;53:651–658.
5. Schuler-Thurner B, Schultz ES, Berger TG, et al. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med.* 2002;195:1279–1288.
6. Lee AW, Truong T, Bickham K, et al. A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. *Vaccine.* 2002; 20(Suppl 4):A8–A22.
7. Alli RS, Khar A. Interleukin-12 secreted by mature dendritic cells mediates activation of NK cell function. *FEBS Lett.* 2004;559:71–76.
8. Mbawuike IN, Fujihashi K, DiFabio S, et al. Human interleukin-12 enhances interferon-gamma-producing influenza-specific memory CD8+ cytotoxic T lymphocytes. *J Infect Dis.* 1999;180:1477–1486.
9. Dredge K, Marriott JB, Todryk SM, et al. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. *Cancer Immunol Immunother.* 2002;51:521–531.
10. Toes RE, Offringa R, Feltkamp MC, et al. Tumor rejection antigens and tumor specific cytotoxic T lymphocytes. *Behring Inst Mitt.* 1994;94: 72–86.
11. Okada N, Mori N, Koretomo R, et al. Augmentation of the migratory ability of DC-based vaccine into regional lymph nodes by efficient CCR7 gene transduction. *Gene Ther.* 2005;12:129–139.
12. Hirao M, Onai N, Hiroishi K, et al. CC chemokine receptor-7 on dendritic cells is induced after interaction with apoptotic tumor cells: critical role in migration from the tumor site to draining lymph nodes. *Cancer Res.* 2000; 60:2209–2217.
13. Kim KD, Choi SC, Kim A, et al. Dendritic cell-tumor coculturing vaccine can induce antitumor immunity through both NK and CTL interaction. *Int Immunopharmacol.* 2001;1:2117–2129.

14. Nishimura T, Nakui M, Sato M, et al. The critical role of Th1-dominant immunity in tumor immunology. *Cancer Chemother Pharmacol.* 2000; 46 (Suppl):S52–S61.
15. Dauer M, Obermaier B, Herten J, et al. Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. *J Immunol.* 2003;170:4069–4076.
16. Nakahara S, Tsunoda T, Baba T, et al. Dendritic cells stimulated with a bacterial product, OK-432, efficiently induce cytotoxic T lymphocytes specific to tumor rejection peptide. *Cancer Res.* 2003;63:4112–4118.
17. Itoh T, Ueda Y, Okugawa K, et al. Streptococcal preparation OK432 promotes functional maturation of human monocyte-derived dendritic cells. *Cancer Immunol Immunother.* 2003;52:207–214.
18. Nukaya I, Yasumoto M, Iwasaki T, et al. Identification of HLA-A24 epitope peptides of carcinoembryonic antigen which induce tumor-reactive cytotoxic T lymphocyte. *Int J Cancer.* 1999;80:92–97.
19. Ito A, Kanto T, Kuzushita N, et al. Generation of hepatitis C virus-specific cytotoxic T lymphocytes from healthy individuals with peptide-pulsed dendritic cells. *J Gastroenterol Hepatol.* 2001;16:309–316.
20. Scandella E, Men Y, Gillessen S, et al. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood.* 2002;100:1354–1361.
21. Kalinski P, Vieira PL, Schuitemaker JH, et al. Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. *Blood.* 2001;97:3466–3469.
22. Brombacher F, Kastelein RA,Alber G. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol.* 2003;24:207–212.
23. Figdor CG, De Vries IJ, Lesterhuis WJ, et al. Dendritic cell immunotherapy: mapping the way. *Nat Med.* 2004;10:475–480.
24. Curti A, Isidori A, Ferri E, et al. Generation of dendritic cells from positively selected CD14+ monocytes for anti-tumor immunotherapy. *Leuk Lymphoma.* 2004;45:1419–1428.
25. Sorg RV, Ozcan Z, Brefort T, et al. Clinical-scale generation of dendritic cells in a closed system. *J Immunother.* 2003;26:374–383.
26. Wong EC, Maher VE, Hines K, et al. Development of a clinical-scale method for generation of dendritic cells from PBMC for use in cancer immunotherapy. *Cytotherapy.* 2001;3:19–29.
27. Moldenhauer A, Nociari MM, Dias S, et al. Optimized culture conditions for the generation of dendritic cells from peripheral blood monocytes. *Vox Sang.* 2003;84:228–236.
28. Kubo S, Takahashi HK, Takei M, et al. EP (E-Prostanoid)2/EP4-receptor-dependent maturation of human monocyte-derived dendritic cells and induction of Th2 polarization. *J Pharmacol Exp Ther.* 2004;9:9–99.
29. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol.* 2004;4:941–952.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Hepatology Research 34 (2006) 156–162
HEPATOLOGY RESEARCH
http://www.elsevier.com/locate/hepres

Reduced expression and functional impairment of Toll-like receptor 2 on dendritic cells in chronic hepatitis C virus infection

Takayuki Yakushijin[a,*], Tatsuya Kanto[a,b], Michiyo Inoue[b], Tsugiko Oze[a], Masanori Miyazaki[a], Ichiyo Itose[a], Hideki Miyatake[a], Mitsuru Sakakibara[a], Noriyoshi Kuzushita[a], Naoki Hiramatsu[a], Tetsuo Takehara[a], Akinori Kasahara[c], Norio Hayashi[a,*]

[a] *Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[b] *Department of Dendritic Cell Biology and Clinical Application, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[c] *Department of General Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*

Received 27 November 2005; received in revised form 28 December 2005; accepted 28 December 2005
Available online 3 February 2006

Abstract

Background: Dendritic cells (DCs) utilize Toll-like receptors (TLRs) to sense virus and initiate immune responses. We aimed at elucidating the roles of TLRs on DCs in hepatitis C virus (HCV) infection.
Methods: Monocyte-derived DCs were obtained from 32 healthy volunteers (HV) and 30 chronically HCV-infected patients (CH). TLR2, TLR3 and TLR4 expressions on immature DCs were quantified by real-time quantitative RT-PCR. We stimulated DCs with specific TLR ligands and examined DC maturation, cytokine production and ability to stimulate allogeneic $CD4^+$ T cells.
Results: TLR2 expression on immature DCs was lower in the CH group, whereas those of TLR3 or TLR4 were not different between the groups. Each TLR ligand induced DC maturation and stimulated them to release comparable levels of IL-12p70, IL-6, IL-10, TNF-α and IFN-β between the groups. TLR2 and TLR4 ligands enhanced DC ability to stimulate T cell proliferation, with the degree due to the TLR2 ligand being lower in the CH group.
Conclusions: In HCV infection, the TLR2 expression on DCs is reduced and TLR2-stimulated DCs show lesser ability to proliferate T cells than healthy counterparts, suggesting that the TLR2 system is involved in HCV-induced immunopathogenesis.

Keywords: Dendritic cell; Hepatitis C virus; Toll-like receptor; Mixed lymphocyte reaction

1. Introduction

Hepatitis C virus (HCV), a single-stranded RNA virus that belongs to the flaviviridae family, is a major causative agent of chronic liver disease [20]. Approximately 80% of newly HCV-infected patients are not able to eradicate HCV, and subsequently develop to a chronically infected state. One of the most important factors involved in HCV persistence is inadequate cell-mediated immune response in hosts. Several studies have demonstrated that a vigorous and sustained $CD4^+$ T cell response is correlated with spontaneous viral clearance in acute HCV infection [10,24]. In chronic HCV infection, the $CD8^+$ T cell response to HCV proteins as well as that of $CD4^+$ T cell was less vigorous and narrowly focused on some restricted epitopes [30,36]. Although the precise mechanism of such impaired T cell responses in HCV infection is yet to be determined, impaired function of dendritic cells (DCs) may be involved, since DCs are the most potent antigen presenting cells (APCs) that regulate immune responses [12]. We previously reported that monocyte-derived DCs (MDDCs)

* Corresponding authors. Tel.: +81 6 6879 3621; fax: +81 6 6879 3629.
E-mail addresses: yakushijin@gh.med.osaka-u.ac.jp (T. Yakushijin), hayashin@gh.med.osaka-u.ac.jp (N. Hayashi).

1386-6346/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.hepres.2005.12.010

from HCV-positive patients are impaired in allogeneic $CD4^+$ T cell stimulation compared with those from healthy volunteers [19]. One of the causes of such DC dysfunction may be the failure of DC to mature in response to some inflammatory stimuli, which may be due to direct HCV infection to DCs [3,25] or the influences of HCV proteins [7] as reported elsewhere.

Toll-like receptors (TLRs) recognize molecular patterns specific to microbial pathogens [1]. DCs show subset-specific expressions of TLRs on their cell surface or endosomal membrane [16,18]. Cumulative reports have shown that DC activation via TLR signaling is prerequisite for the subsequent induction of vigorous T cell responses. Some viruses have been shown to interact with TLRs or their downstream molecules and interfere with the signaling cascade [9,13]. These reports suggest that the alteration of TLR-mediated signals is one of the mechanisms of virus-induced immune modulation. We thus hypothesize that HCV infection influences the profiles of TLR expressions as well as their functions in DCs resulting in aberrant DC-mediated immune response. To address this issue, we compared the expressions of TLRs on MDDCs between patients with chronic HCV infection and healthy subjects. To study their functions, we stimulated MDDCs with TLR-specific ligands and then examined DC phenotypes, cytokine release and DC-induced T cell responses.

2. Materials and methods

2.1. Subjects

Thirty-two healthy volunteers (HV) and 30 patients with chronic hepatitis C (CH) followed at Osaka University Hospital (Osaka, Japan) were enrolled in the present study. Informed consent was obtained from each patient or donor included in the study, and the study protocol conformed to The Code of Ethics of the World Medical Association for experiments involving humans. The healthy volunteers include buffy coats provided from the Osaka Red Cross Blood Center (Osaka, Japan) and they were confirmed to be negative for hepatitis C virus, hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The characteristics of patients are shown in Table 1. All patients were confirmed to be positive for both serum anti-HCV antibody and HCV RNA but were negative for other viral infections, including HBV and HIV.

Table 1
The characteristics of HCV-infected patients enrolled in the present study

Gender (male/female)	21/9
Age (years)	57.1 ± 1.9
Serotype (1/2/N.D.)	23/0/7
HCV-RNA (Meq/mL)	8.1 ± 1.7
Serum ALT (IU/L)	62.7 ± 9.3

Data were expressed as mean ± standard error. N.D.: not determined.

2.2. Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were purchased from PeproTech (Rocky Hill, NJ). Palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) was purchased from InvivoGen (San Diego, CA). Polyinosine-polycytidylic acid (poly(I:C)) and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma (St. Louis, MO).

2.3. Generation of MDDCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by density gradient centrifugation using Ficoll–Hypaque. $CD14^+$ monocytes were purified from PBMCs using the MACS system (Miltenyi Biotec, Gladbach, Germany) with anti-CD14 antibody-conjugated microbeads. MDDCs were generated from $CD14^+$ cells (purity was >96%) by the culture in DC media (Isocove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY)) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L non-essential amino acid) containing 25 ng/mL GM-CSF and 10 ng/mL IL-4 for 7 days at 37 °C in 5% CO_2. On day 4, half of the culture supernatants were replaced with fresh media containing equal concentrations of cytokines. On day 7, MDDCs were used to analyze the expression of TLRs without any stimulus. The remaining cells were stimulated with selective TLR ligands, Pam3CSK4 for TLR2, poly(I:C) for TLR3 and LPS for TLR4, respectively. After 24 h of incubation, the supernatants were harvested for the measurement of cytokines by enzyme-linked immunosorbent assay (ELISA). The cells were then subjected to immunophenotyping and RNA extraction.

2.4. Flow cytometric analysis

The phenotypes of MDDCs were analyzed by FACS Calibur (BD Biosciences, San Jose, CA). For the staining, MDDCs were incubated with specific antibodies for 15 min at room temperature in phosphate buffered saline containing 2% of bovine serum albumin and 0.1% of sodium azide. The following FITC-, PE-, PerCP-, or PC5-conjugated anti-human monoclonal antibodies were used: CD11c (KB90, Dako Cytomation, Glostrup, Denmark), HLA-DR (L243, BD Biosciences), CD80 (L307.4, BD Biosciences), CD86 (IT2.2, BD Biosciences) and CD83 (HB15a, Beckman Coulter, Flullerton, CA). The expression of each molecule was expressed by mean fluorescence intensity (MFI), which was determined using CellQuest software (BD Biosciences).

2.5. Real-time quantitative PCR

Total RNA from MDDCs was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the

manufacturer's instructions. Total RNA (0.3–1 μg) was reverse transcribed in a 20 μL volume using SuperScript III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Random hexamers were added as primers. The mRNA levels were evaluated using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). For the quantification of TLR2, TLR3, TLR4 and IFN-β, we used ready-to-use assays (Assay-on-Demands Gene Expression Products, Applied Biosystems), according to the manufacturer's instructions. All of the reagents used for PCR were purchased from Applied Biosystems. All of the reactions were performed in duplicate. The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. We identified a calibrator sample from healthy volunteers and the other samples expressed relative values to the calibrator. To normalize the amount of total RNA added to each reaction mixture, we quantified β-actin mRNA from each sample as a control of internal RNA and corrected all values using this.

2.6. ELISA

The cytokines released from MDDCs stimulated with TLR ligands were measured by means of ELISA with matched antibody pairs for human IL-12p70, IL-6, IL-10, TNF-α (Endogen, Woburn, MA), according to the manufacturer's instructions (The ranges of the detection were 4–1000 pg/mL for IL-12p70, IL-10 and TNF-α and 31–1000 pg/mL for IL-6).

2.7. Allogeneic mixed lymphocyte reaction (MLR)

Responder $CD4^+$ T cells were purified from PBMCs of one healthy volunteer using the MACS system with anti-CD4 monoclonal antibody-conjugated microbeads (Miltenyi Biotec). After 7 days of culture, the graded numbers of MDDCs stimulated with each TLR ligand for 6 h were co-cultured with 1×10^5 $CD4^+$ T cells for 5 days. In the final 16–20 h of co-culture, 1 μCi/well of $[^3H]$-thymidine (ICN Biomedicals, Costa Mesa, CA) was pulsed. The uptake of $[^3H]$-thymidine to T cells was measured using a β-counter (Wallac, Gaithersburg, MD).

2.8. Statistical analysis

The values were compared by Mann–Whitney *U*-test and Wilcoxon signed rank test, using StatView 5.0 software (SAS Institute, Cary, NC). A *p*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. The expression of TLR2 was lower in CH

In this study, we obtained highly pure (>98%) immature MDDCs ($CD14^-$, $CD11c^+$ and $HLA\text{-}DR^+$ cells) after 7 days of culture from both HV and CH groups. It is known that immature MDDCs express transcripts spanning from TLR1 to TLR8 [16,18]. We examined TLR2, TLR3 and TLR4 as representatives of TLR to clarify their roles in MDDCs in HCV infection. We evaluated the expression of TLRs using real-time quantitative RT-PCR. The relative amounts of TLR2 in MDDCs from CH were lower than those from HV. In contrast, the relative amounts of TLR3 and TLR4 revealed no significant differences between the groups (Fig. 1). We investigated the correlation between each TLR expression and clinical parameters among HCV-infected patients. Both HCV viral load and serum ALT level were not correlated with each TLR expression (Data not shown).

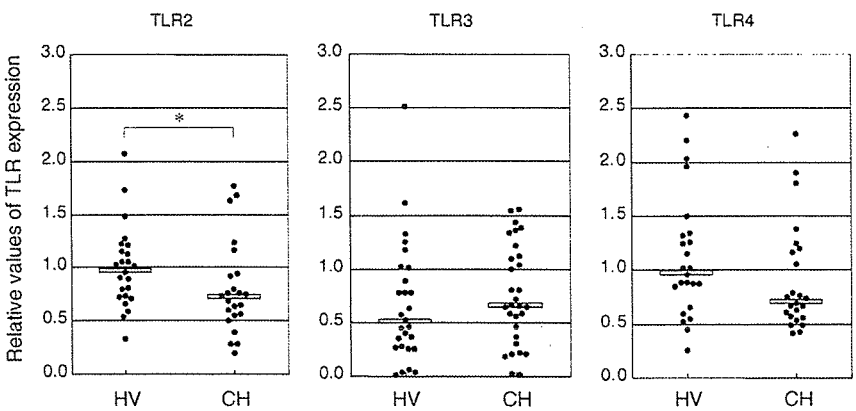

Fig. 1. TLR2 expression in MDDCs from HCV-infected patients is lower than those from healthy donors. The expressions of TLR2, TLR3 and TLR4 mRNA in day 7 MDDC were quantified by real-time quantitative RT-PCR as described in Section 2. The values were determined as the ratio of the results between the samples and a calibrator sample from healthy volunteers after they had been corrected for the amount of β-actin mRNA from each sample as an internal control. The horizontal bars indicate the median. $^*p < 0.05$ by Mann–Whitney *U*-test. HV: healthy volunteers; CH: chronic hepatitis C patients.

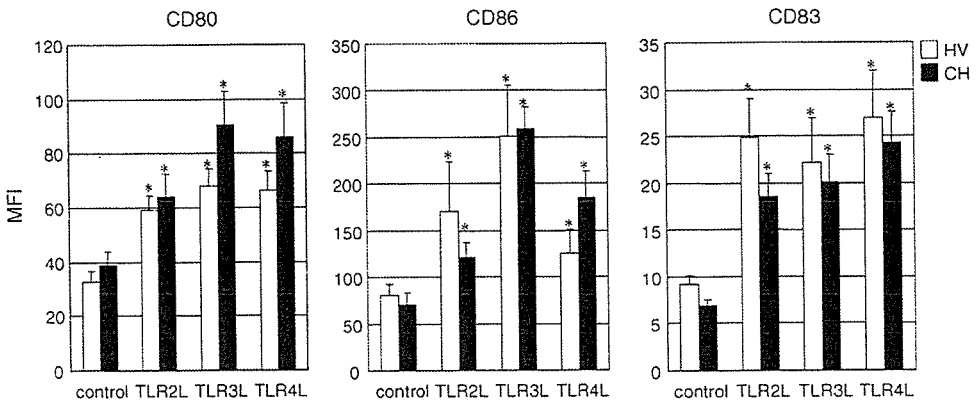

Fig. 2. TLR ligands induce comparable levels of MDDC maturation in healthy donors and HCV-infected patients. The expression of CD80, CD86 and CD83 on MDDC were analyzed by flow cytometry before and after the stimulation with TLR ligands. The values were expressed as mean fluorescence intensity (MFI). The MFI of each marker is represented as the mean + standard error (S.E.) of six healthy donors (open bars) and six patients (closed bars). $^{*}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH. as the same in Fig. 1.

3.2. Immature MDDC from CH matured on stimulation with TLR ligands

To examine the capacity of TLR ligands to induce MDDC maturation, we performed flow cytometric analysis of cell surface markers (CD80, CD86 and CD83). The cells from HV and CH displayed the characteristic of immature MDDC in the absence of any stimulus (Fig. 2). Pam3CSK4, poly(I:C) and LPS significantly up-regulated these markers on MDDCs regardless of HCV infection. The expressions of all maturation markers did not statistically differ between the groups.

3.3. The ability of cytokine production in response to TLR ligands was preserved in MDDC from CH

Mature MDDCs release inflammatory cytokines in response to TLR ligands. We thus examined the production of IL-12p70, IL-6, IL-10 and TNF-α or the expression of IFN-β in TLR-stimulated MDDCs. Each TLR ligand induced the production of IL-10, IL-6 and TNF-α from MDDCs, with the degree induced by LPS being the most significantly (Fig. 3). As for IL-12p70, poly(I:C) strongly induced the production, whereas Pam3CSK4 and LPS did not. In the preliminary experiments, we examined the kinetics of IFN-β expression in TLR-stimulated MDDC. In both HV and CH groups, the IFN-β expression in MDDC showed a peak at 3 h after the stimulation with TLR ligands (data not shown). Thus, we compared its expression at this point in the following experiments. Only poly(I:C) was able to stimulate MDDC to express IFN-β (Fig. 4). Although there was a trend that TLR3L-stimulated MDDC from the patients released more IL-12p70, TNF-α than those from healthy donors, the data for the cytokine production and IFN-β expression were not statistically different between the groups.

3.4. TLR2 specific ligand strongly enhanced the ability of MDDC to stimulate T cell proliferation in MDDC

The ability of DC to stimulate $CD4^+$ T cell proliferation is one of the most important characteristics of DCs as professional APCs. We thus examined allogeneic MLR by MDDC stimulated with various TLR ligands (Fig. 5). In both HV and CH groups, Pam3CSK4 and LPS enhanced the MDDC ability to stimulate allogeneic $CD4^+$ T cell proliferation compared to those without stimulation. In the CH group, the degree of $CD4^+$ T cell proliferation with Pam3CSK4-stimulated MDDCs was significantly lower than those from the HV group. In contrast, poly(I:C) did not enhance MDDC capacity to stimulate $CD4^+$ T cells either in the CH or HV group.

4. Discussion

TLRs recognize various pathogens and are able to initiate the chain of immune responses by linking innate and adaptive immune systems. Several lines of evidence have demonstrated that TLR-mediated signaling which leads to the induction of type-I interferon plays an important role in the eradication of intracellular pathogens, including viruses [15,17,21]. DCs express distinct TLRs according to the differences in their ontogeny and maturation stage [16,18]. In general, TLR ligands induce DC maturation and enhance their ability to produce inflammatory cytokines [4,29,32] and to stimulate T cell responses [4,27], thus contributing to viral eradication. However, it has not been clarified whether TLR expression on DC as well as their function is preserved or not in disease conditions, such as HCV infection.

In the present study, we demonstrated that TLR2 expression on immature MDDC from HCV-infected patients is lower than those from healthy subjects. As for the correlation

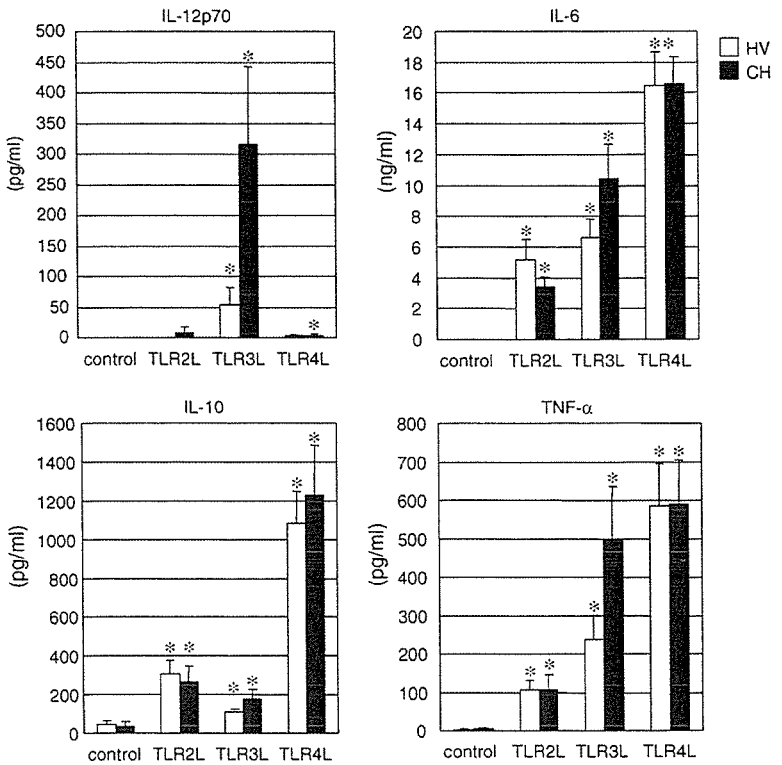

Fig. 3. TLR ligands stimulate MDDC to secrete comparable amounts of inflammatory cytokines between samples from healthy donors and HCV-infected patients. The concentrations of IL-12p70, IL-6, IL-10 and TNF-α in media from DC cultured with TLR ligands for 24 h were measured by ELISA. The data from 16 healthy donors (open bars) and 18 patients (closed bars) are shown as the mean + S.E. $^{*}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH, as the same in Fig. 1.

with TLR expression and DC functions, TLR2-stimulated DC from HCV-infected patients showed lesser ability to proliferate allogeneic $CD4^{+}$ T cells than those from the healthy counterparts. In contrast, TLR3 and TLR4 expressions on MDDC from the patients and their functions in DC maturation are comparable with those from healthy donors.

In spite of recent research progress on the innate immune system, it has been largely unknown how TLR expression is regulated. In several disease conditions, increased TLR2 expression has been reported, which may be due to inflammatory mediators derived from inflamed tissue where virus replication or autoimmunity occurs. It is considered that IFN-γ, IL-12 or IL-18 are responsible for TLR2 up-regulation on epithelial cells [5] or PBMCs [28]. With regard to TLR2 down-regulation, there is one report on all-trans retinoic acid [23]. Why TLR2 expression is decreased on MDDC from HCV-infected patients is currently unknown. It is conceivable that direct HCV infection to DC or the influence of HCV-derived proteins is involved in TLR2 down-regulation, since these are mechanisms reported to lead to DC disability in HCV infection [3,7,25].

TLR2 ligand induced phenotypic as well as functional DC maturation as judged by up-regulation of co-stimulators and enhancement of MLR. MLR is generally accepted as one of the reliable values for assessing comprehensive DC function [11,33]. However, the precise factors dictating the magnitude of MLR are still under debate. Plausible MLR determinants are the expressions of CD86 on DC [37] or DC-derived cytokines including IL-12 [14,26]. In the present study, the expression of CD86 and CD83 on MDDCs stimulated with Pam3CSK4 tended to be lower in CH group than those in HV group, though it failed to reach statistical significance. As for DC-derived cytokines, we could not measure bioactive IL-12p70 produced by MDDCs stimulated with Pam3CSK4, of which level is much less than IL-12 p40 as reported elsewhere [34]. In addition, we did not observe any difference in IL-10 levels, which is reported to subvert T cell proliferation [6]. Therefore, it is still unclear about the precise mechanisms of the lower level of MLR induced by TLR2-stimulated MDDC in patients compared to those in healthy subjects. Lesser degree of CD86 expression on MDDC in HCV-infected patients may be involved. Further examination is still needed to disclose the involvement of other factors in MLR impairment with HCV-DC.

In this study, no significant enhancement in MLR was obtained with TLR3 ligand-stimulated MDDC regardless of HCV infection, which is in clear contrast with previous reports [22,35]. Such contradictory results may be due to the difference in duration of the poly(I:C) treatment. We matured

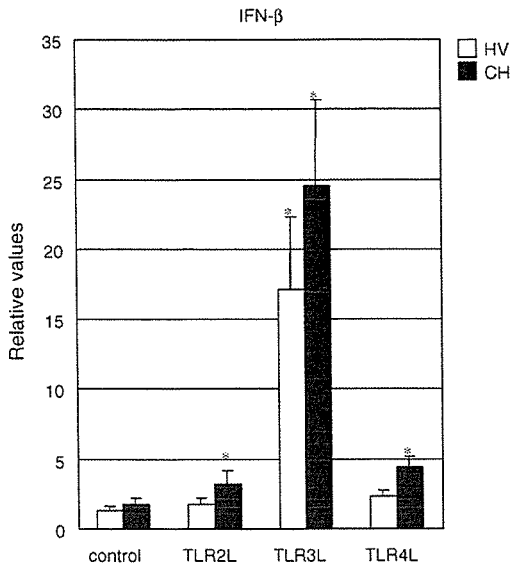

Fig. 4. TLR ligands induce comparable levels of IFN-β mRNA expression in MDDC from healthy donors and HCV-infected patients. The expression of IFN-β in MDDC was analyzed by real-time quantitative RT-PCR 3 h after stimulation with TLR ligands as described in Section 2. The values of IFN-β expression were determined as the same in TLR expression analyses. The results of 11 healthy donors (open bars) and 11 patients (closed bars) are shown as the mean + S.E. $^{*}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH, as the same in Fig. 1.

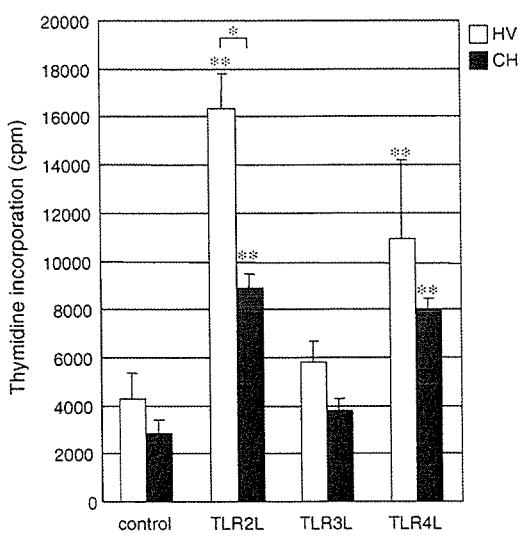

Fig. 5. MDDCs from HCV-infected patients fail to gain comparable ability with those from healthy counterparts to stimulate allogeneic CD4+ T cell proliferation in response to TLR2 ligand. After MDDC had been stimulated with TLR ligands for 6 h, allogeneic CD4+ T cells were co-cultured with DC for 5 days (DCs:T cells = 1:20). The CD4+ T cell proliferation was determined by their uptake of [^{3}H]-thymidine as described in Section 2. The amounts of thymidine incorporation to T cells stimulated with DC from six healthy donors (open bars) and six patients (closed bars) are expressed as the mean + S.E. $^{*}p < 0.05$ by Mann–Whitney U test. $^{**}p < 0.05$ vs. control by Wilcoxon signed rank test. HV and CH, as the same in Fig. 1.

MDDCs by 6 h stimulation of poly (I:C), which was shorter than the condition performed in other studies.

Evoking of the T cell response against allo-antigens has been shown to parallel a successful T cell response against specific antigens [2]. Thus, impaired MLR with TLR2-stimulated DC from HCV-infected patients may be related to impaired ability of HCV-DC to mount a vigorous T cell response, as reported by several groups including us [3,19,31]. Recently, Dolganiuc et al. [8] reported that HCV core and NS3 proteins were directly associated with TLR2 on monocytes and subsequently stimulated them to release TNF-α, suggesting some role of TLR2-mediated signals in the pathogenesis of HCV infection. To elucidate this, further work is needed to examine the association between TLR2 and other HCV-related proteins, and whether the expression and function of TLR2 are changed when HCV is eradicated by IFN-α-based therapy.

In conclusion, in HCV infection, TLR2 expression on DC is reduced and DC maturation via TLR2 fails to induce a level of CD4+ T cell proliferation comparable to that observed with DC those from healthy subjects, suggesting that the TLR2 system is involved in HCV-induced immunopathogenesis.

Acknowledgements

This work was supported by the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Science and Culture of Japan and the Organization for Pharmaceutical Safety and Research.

References

[1] Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000;406:782–7.
[2] Awdeh ZL, Alper CA, Fici DA, Ronco II P, Yunis EJ. Predictability of alloreactivity among unrelated individuals: role for HLA-DPB1. Tissue Antigens 1995;46:180–6.
[3] Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology 2001;120:512–24.
[4] Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med 1999;189:821–9.
[5] Cook EB, Stahl JL, Esnault S, Barney NP, Graziano FM. Toll-like receptor 2 expression on human conjunctival epithelial cells: a pathway for Staphylococcus aureus involvement in chronic ocular proinflammatory responses. Ann Allergy Asthma Immunol 2005;94:486–97.
[6] Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. J Immunol 2001;166:4312–8.
[7] Dolganiuc A, Kodys K, Kopasz A, et al. Hepatitis C virus core and non-structural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. J Immunol 2003;170:5615–24.
[8] Dolganiuc A, Oak S, Kodys K, et al. Hepatitis C core and non-structural 3 proteins trigger Toll-like receptor 2-mediated pathways and inflammatory activation. Gastroenterology 2004;127:1513–24.

[9] Equils O, Shapiro A, Madak Z, Liu C, Lu D. Human immunodeficiency virus type 1 protease inhibitors block Toll-like receptor 2 (TLR2)- and TLR4-Induced NF-kappaB activation. Antimicrob Agents Chemother 2004;48:3905–11.
[10] Ferrari C, Valli A, Galati L, et al. T-cell response to structural and non-structural hepatitis C virus antigens in persistent and self-limited hepatitis C virus infections. Hepatology 1994;19:286–95.
[11] Guidos C, Sinha A, ALee KC. Functional differences and complementation between dendritic cells and macrophages in T-cell activation. Immunology 1987;61:269–76.
[12] Hart DN. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 1997;90:3245–87.
[13] Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. Science 2004;303:1526–9.
[14] Hill JA, Ichim TE, Kusznieruk KP, et al. Immune modulation by silencing IL-12 production in dendritic cells using small interfering RNA. J Immunol 2003;171:691–6.
[15] Ito T, Amakawa R, Kaisho T, et al. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. J Exp Med 2002;195:1507–12.
[16] Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 2001;31:3388–93.
[17] Kadowaki N, Antonenko S, Liu YJ. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c$^-$ type 2 dendritic cell precursors and CD11c$^+$ dendritic cells to produce type I IFN. J Immunol 2001;166:2291–5.
[18] Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. J Exp Med 2001;194:863–9.
[19] Kanto T, Hayashi N, Takehara T, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol 1999;162:5584–91.
[20] Kiyosawa K, Sodeyama T, Tanaka E, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12:671–5.
[21] Krug A, Rothenfusser S, Hornung V, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol 2001;31:2154–63.
[22] Lee AW, Truong T, Bickham K, et al. A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. Vaccine 2002;20(Suppl. 4):A8–22.
[23] Liu PT, Krutzik SR, Kim J, Modlin RL. Cutting edge: all-trans retinoic acid down-regulates TLR2 expression and function. J Immunol 2005;174:2467–70.
[24] Missale G, Bertoni R, Lamonaca V, et al. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J Clin Invest 1996;98:706–14.
[25] Navas MC, Fuchs A, Schvoerer E, Bohbot A, Aubertin AM, Stoll-Keller F. Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. J Med Virol 2002;67:152–61.
[26] Nishioka Y, Wen H, Mitani K, et al. Differential effects of IL-12 on the generation of alloreactive CTL mediated by murine and human dendritic cells: a critical role for nitric oxide. J Leukoc Biol 2003;73:621–9.
[27] Pulendran B, Kumar P, Cutler CW, Mohamadzadeh M, Van Dyke T, Banchereau J. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. J Immunol 2001;167:5067–76.
[28] Radstake TR, Roelofs MF, Jenniskens YM, et al. Expression of Toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. Arthritis Rheum 2004;50:3856–65.
[29] Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem 2001;276:37692–9.
[30] Rehermann B, Chang KM, McHutchinson J, et al. Differential cytotoxic T-lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. J Virol 1996;70:7092–102.
[31] Rollier C, Drexhage JA, Verstrepen BE, et al. Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment. Hepatology 2003;38:851–8.
[32] Rouas R, Lewalle P, El Ouriaghli F, Nowak B, Duvillier H, Martiat P. Poly(I:C) used for human dendritic cell maturation preserves their ability to secondarily secrete bioactive IL-12. Int Immunol 2004;16:767–73.
[33] Steinman RM, Gutchinov B, Witmer MD, Nussenzweig MC. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. J Exp Med 1983;157:613–27.
[34] Thoma-Uszynski S, Kiertscher SM, Ochoa MT, et al. Activation of Toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. J Immunol 2000;165:3804–10.
[35] Verdijk RM, Mutis T, Esendam B, et al. Polyriboinosinic-polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. J Immunol 1999;163:57–61.
[36] Wedemeyer H, He XS, Nascimbeni M, et al. Impaired effector function of hepatitis C virus-specific CD8$^+$ T cells in chronic hepatitis C virus infection. J Immunol 2002;169:3447–58.
[37] Young JW, Koulova L, Soergel SA, Clark EA, Steinman RM, Dupont B. The B7/BB1 antigen provides one of several costimulatory signals for the activation of CD4$^+$ T lymphocytes by human blood dendritic cells in vitro. J Clin Invest 1992;90:229–37.