Fig. 1. Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. Thirty-two patients received the therapy, but seven dropped out due to various adverse effects. Among the 25 who completed the therapy, 11 achieved sustained virological response, 11 were transient responders, and 3 were non-responders. The early responders were defined as those who showed a reduction in HCV RNA quantity to an undetectable level by qualitative PCR at Week 12 of the therapy. According to this criterion, 11 patients were early responders and were further categorized into 7 sustained virological response (sustained virological responders with early response) and 4 transient response (transient responders with early response). Of the other 14 patients who were not early responders, 4 were sustained virological responders, 7 were transient responders, and 3 were non-responders.

Non-Sustained Virological Responders Had a Lower MLR Ratio Than Sustained Virological Responders

In order to clarify whether the frequency and function of immune cells are involved in the outcomes of the combination therapy, these parameters were compared between sustained virological responders and non-sustained virological responders, including transient responders and no responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and non-sustained virological responders (Fig. 2A). As for the changes of dendritic cell subsets during the therapy, frequencies of both plasmacytoid dendritic cells and myeloid dendritic cells at each time point did not differ between sustained virological responders and non-sustained virological responders (Fig. 2B,C). The percentages of NK cells in non-sustained virological

responders tended to be higher than those in sustained virological responders from Weeks 4–48, which did not reach statistical significance $(P = 0.0533$ ANOVA) (Fig. 2F). The frequencies of Th1 and Th2 did not differ between these two groups (Fig. 2G,H). As for dendritic cell function, dendritic cells from the non-sustained virological responders showed a lower MLR ratio than those from the sustained virological responders at the end $(P < 0.01)$ and at 4 weeks after the completion of therapy $(P < 0.005)$ (Fig. 3). These results show that lesser ability of dendritic cells at the end of treatment may be related to non-sustained virological response.

Transient Responders Had a Lower MLR Ratio in Dendritic Cell Function Than Sustained Virological Responders in the Course of Combination Therapy

In order to elucidate if the above-mentioned immunological markers are related to virological relapse, a

Fig. 2. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (A), during the combination therapy (B, C, F–H) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (D, E) were determined as described in Materials and Methods, which were compared between sustained virological responders and non-sustained virological responders. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict non-sustained virological responders. The results are expressed as the mean ± SEM of 11 sustained virological responders and 14 non-sustained virological responders. PBMC, peripheral blood mononuclear cells; NK, natural killer.
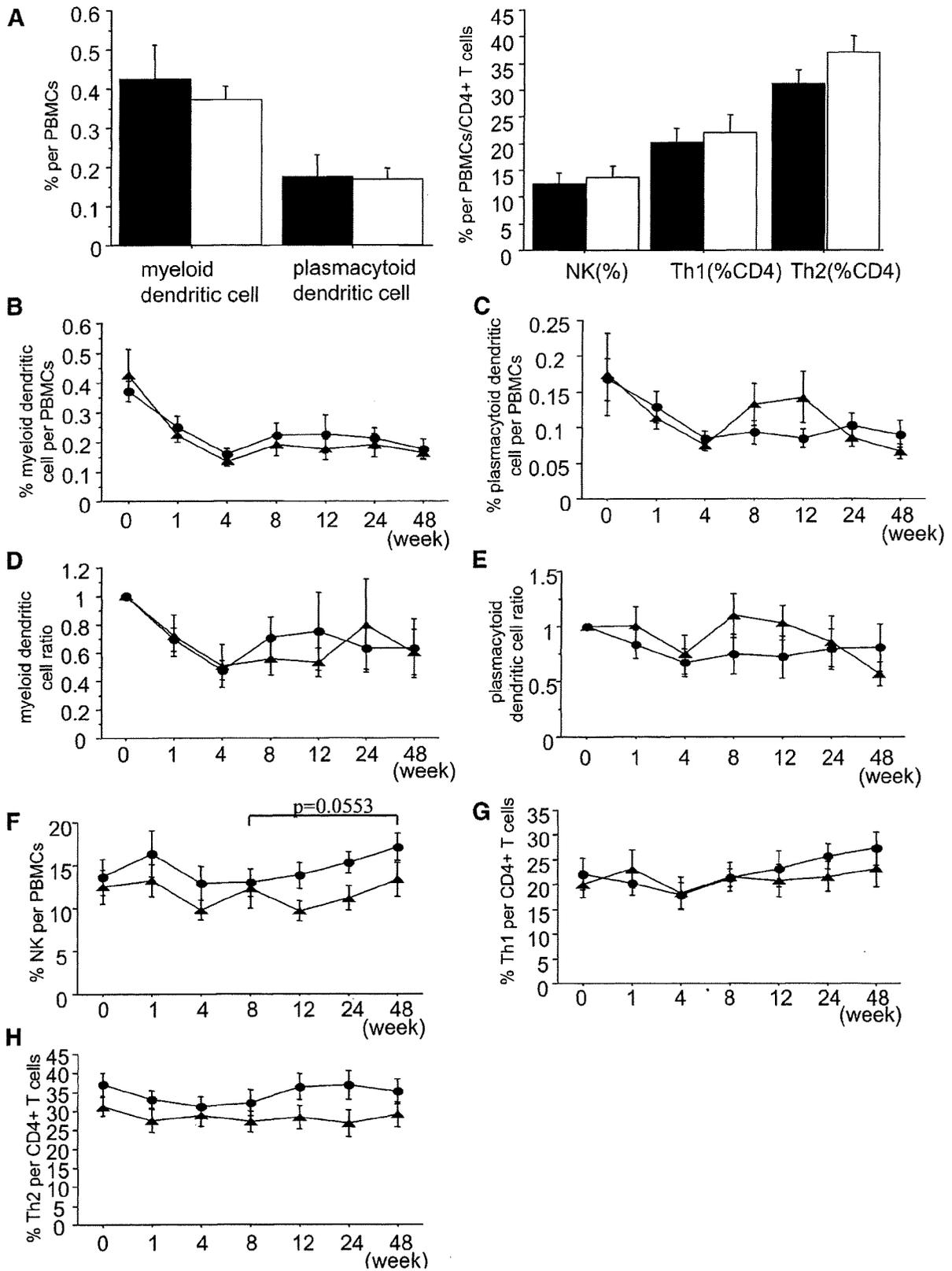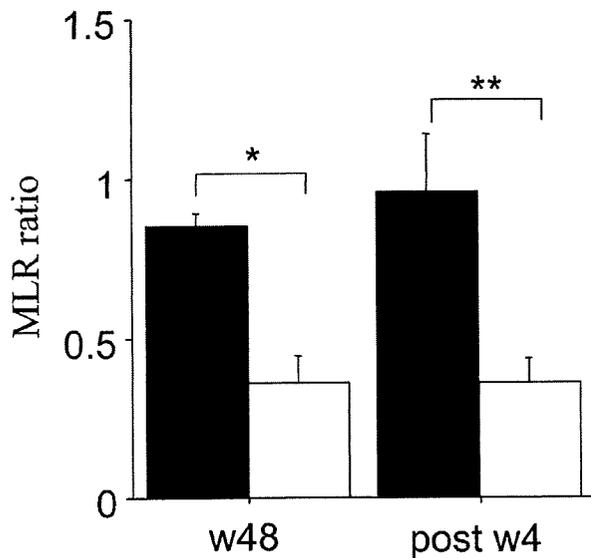
Fig. 2.

Fig. 3. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined from the counts per minute (cpm) of ^{3}H-thymidine incorporated into CD4$^+$ T cells at T cell/dendritic cell ratio of 10/1. The results are expressed as the mean $\pm$ SEM of 11 sustained virological responders and 14 non-sustained virological responders. Black bars indicate sustained virological responders and white bars indicate non-sustained virological responders. *$P < 0.01$, **$P < 0.005$.

comparison was undertaken between sustained virological responders and transient responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and transient responders (Fig. 4A).

The percentages of myeloid dendritic cells and plasmacytoid dendritic cells were not different between the sustained virological responders and transient responders at each time point (Fig. 4B,C). The transient responders tended to show a lower plasmacytoid dendritic cell ratio than sustained virological responders from Weeks 1–12 ($P = 0.0553$, ANOVA) (Fig. 4E), suggesting that plasmacytoid dendritic cell is likely to decrease in the early phase in transient responders whereas those in sustained virological responders tend to be maintained. By contrast, no difference was observed in the myeloid dendritic cell ratio between the groups (Fig. 4D). The percentages of NK cells in transient responders were significantly higher than those in sustained virological responders from

Weeks 8–48 ($P < 0.05$) (Fig. 4F). The frequencies of Th1 or Th2 at each point during therapy did not differ between the sustained virological responders and transient responders (Fig. 4G,H).

With regard to the dendritic cell function, the transient responders showed a lower MLR ratio than the sustained virological responders from Weeks 4–48 after the end of the therapy ($P < 0.05$) (Fig. 5). These results suggest that sustained impairment of dendritic cell function at the end and after the treatment may be related to the virological relapse after cessation of the therapy.

Early-Phase Decline of Plasmacytoid Dendritic Cell Frequency and Sustained Impairment of Dendritic Cell Ability Are Related to Transient Response in the Combination Therapy Even in Patients Who Lost Serum HCV RNA at Week 12 of the Treatment

In order to estimate more precisely the involvement of immunological markers in the outcomes of the combination therapy, we examined the above-mentioned parameters in patients who attained negative serum HCV RNA at Week 12 (early response group), as they were considered to be comparable with respect to the virological response to the therapy. Among 11 patients who were clear of serum HCV at Week 12, 7 were categorized into sustained virological response (sustained virological responders with early response) and the remaining 4 into transient response (transient responders with early response) (Fig. 1). Among patients with early response, the pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, Th1, Th2, and NK cells (Fig. 6A) and those at any points during the therapy did not differ between sustained virological responders and transient responders (Fig. 6B,C,F–H). The plasmacytoid dendritic cell ratios in transient responders were lower than those in sustained virological responders from Weeks 1–12 ($P < 0.05$, ANOVA) (Fig. 6E), whereas the myeloid dendritic cell ratio did not differ between the groups (Fig. 6D).

As for MLR, dendritic cells from the transient responders showed a lower MLR ratio than those from the sustained virological responders at the end and at 4 weeks after the completion of therapy (Fig. 7) ($P < 0.001$).

DISCUSSION

In the PEG-IFNα and ribavirin therapy for chronic hepatitis C, viral and host factors are critically involved in the efficacy of treatment. As for viral factors, HCV

Fig. 4. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (A), during the combination therapy (B, C, F–H), and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (D, E) were determined as described in Materials and Methods, which were compared between sustained

virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean $\pm$ SEM of 11 sustained virological responders and 11 transient responders. PBMC, NK are shown in Figure 2. *$P < 0.05$ (sustained virological responders vs. transient responders).
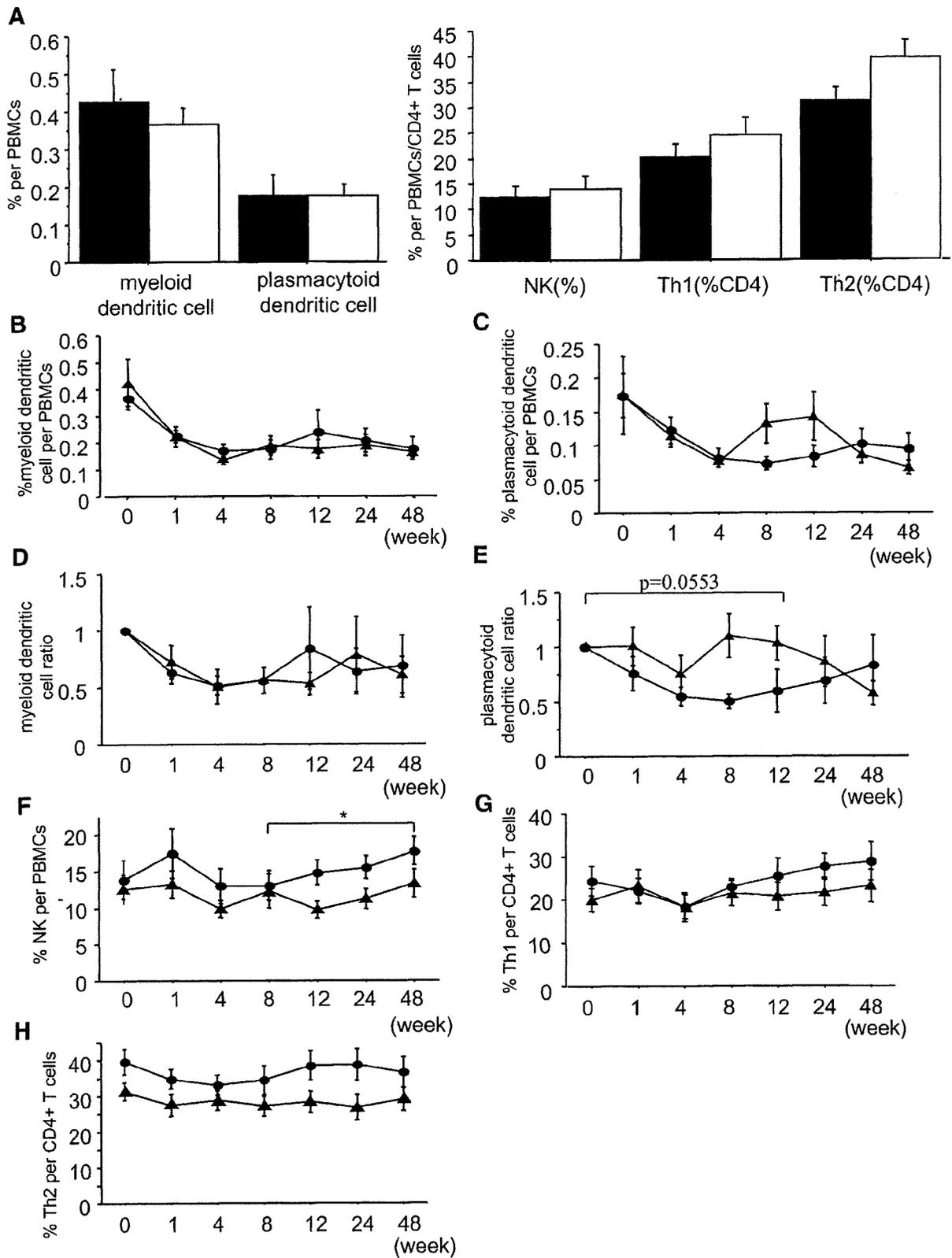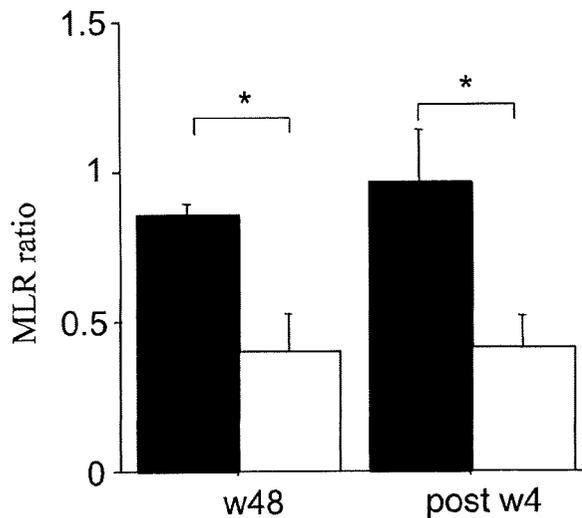
Fig. 4.

Fig. 5. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of 11 sustained virological responders and 11 transient responders. Black bars indicate sustained virological responders and white bars indicate transient responders. $*P < 0.05$.

virological responders and transient responders, transient responders exhibited (1) lower plasmacytoid dendritic cell ratio, (2) higher NK cell frequency, and (3) impaired dendritic cell function than sustained virological responders. Of particular interest were the findings of a lower plasmacytoid dendritic cell ratio as well as lower MLR even in transient responders with early response compared to sustained virological responders with early response. Since patients with early response are defined as those who showed negative serum HCV RNA at Week 12, they are considered to be similar in virological response to the combination therapy. Thus, such parameters could serve as immunological markers for virological relapse, presumably being independent of the early virological response.

In general, homeostasis of blood cell number is regulated by their life span and their recruitment from the bone marrow to circulating blood. A reduction of blood cell numbers is frequently observed in patients who are treated with PEG-IFNα and ribavirin combination therapy, which may be due to bone marrow suppression, enhancement of cellular apoptosis, or alteration of localization. However, the dynamics of dendritic cell subsets or NK cells under combination therapy is yet to be clarified. Some investigators have reported that the frequency or the absolute number of blood dendritic cell is dynamically changed by various stresses, such as infection [Hotchkiss et al., 2002] or surgery [Ho et al., 2001]. The present study showed that reduction of plasmacytoid dendritic cells after the introduction of combination therapy is much greater in the transient responders than in the sustained virological responders. IFNα is reported to act as a regulatory factor on CD11c⁻ dendritic cells to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [Ito et al., 2001]. Thus, patients who respond well to IFNα, as demonstrated by better plasmacytoid dendritic cell survival during the treatment, are likely to have better chances to eradicate HCV. Limited information is available about the factors influencing the number of NK cells. In chronic HCV infection, it has been reported that the progression of liver disease is associated with a decrease of peripheral as well as liver-residing NK cells [Kawarabayashi et al., 2000]. It is plausible that the lower frequency of peripheral NK cells in the sustained virological responders compared to the transient responders, as shown in this study, may be related to the accumulation of NK cells in the liver, where they presumably produce IFNγ to suppress HCV replication. Further study is needed to disclose the reasons for the dynamics of these cells being related to the virological response in the combination therapy.

genotypes and baseline HCV RNA titers are major determinants dictating therapeutic outcomes. In addition, failure of rapid decline in serum HCV RNA from the beginning of the treatment, i.e., non-early virological response, has been used as a negative predictor for sustained virological response. Alternatively, the enhancement of immunity has been implicated to play a key role in the successful responses in PEG-IFNα and ribavirin therapy. However, it is yet to be determined which parameters are practically feasible for the assessment of treatment-induced immune responses correlating with therapeutic efficacy.

In the present study, it was determined whether the frequencies of dendritic cells, NK cells, Th1 and Th2 cells, as well as dendritic cell function in patients are related to the outcome of the PEG-IFNα and ribavirin therapy. By comparing these markers in the course of the treatment between sustained virological responders and non-sustained virological responders, it was demonstrated that non-sustained virological responders showed impaired dendritic cell function in MLR than sustained virological responders. When the analyses were extended to comparison between sustained

Fig. 6. Pretreatment frequency of blood cells and changes during 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (A), during the combination therapy (B, C, F–H) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (D, E) were determined as described in Materials and Methods, which were compared between

sustained virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean±SEM of seven sustained virological responders with early response and four transient responders with early response. PBMC, NK are shown in Figure 2. $*P < 0.05$ (sustained virological responders vs. transient responders).
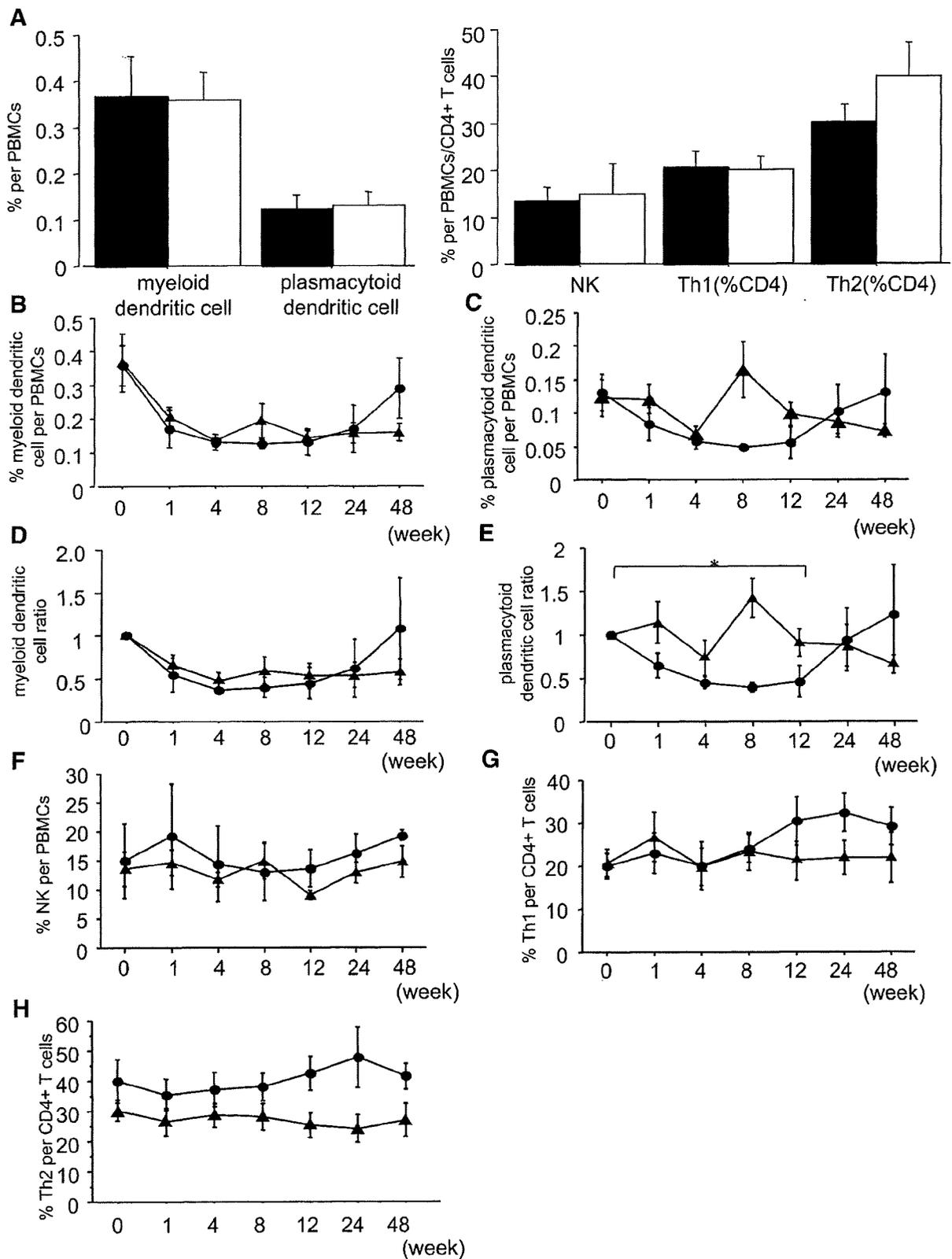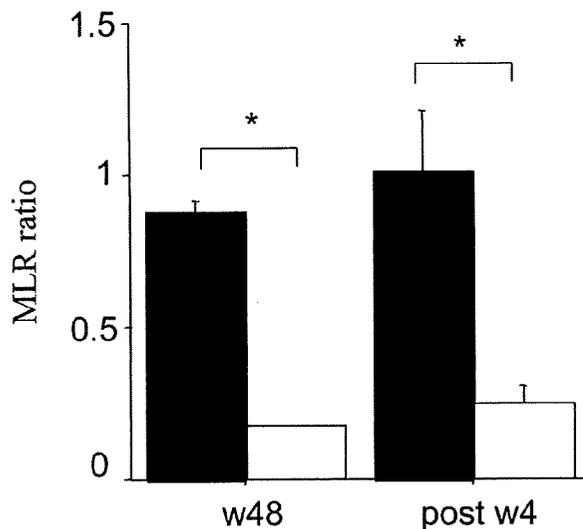
Fig. 6.

Fig. 7. Allostimulatory activity of dendritic cells in the patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. At the end of treatment (Week 48) and at Week 4 after the completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of seven sustained virological responders with early response and four transient responders with early response. Black bars indicate sustained virological responders and white bars indicate transient responders, respectively. *P < 0.05.

restoration of dendritic cell function in non-sustained virological responders or transient responders compared to sustained virological responders.

In summary, it was shown that the frequencies of plasmacytoid dendritic cells or NK cells and dendritic cell function might be related to the outcomes of the combination therapy. Since the present study was performed with a relatively small number of patients, a greater number of patients should be examined in order to validate the feasibility of using these as immunological markers of relapse. The prediction of virological non-response or relapse during therapy can help improve the clinical outcomes of treated patients, as prolongation of combination therapy offers potential relapsers a better chance of sustained virological response by suppressing HCV reappearance.

In the present study, non-sustained virological responders or transient responders showed a lesser capacity for dendritic cell function than sustained virological responders at the end and after cessation of the therapy. Even in the patients who lost serum HCV RNA at Week 12, the dendritic cell function was lower in transient responders than sustained virological responders. One of the mechanisms of impaired dendritic cell function in non-sustained virological responders or transient responders may be residual HCV both in serum and in cells. It is reported that the relapse rate was higher in the patients who were positive for HCV RNA by sensitive transcription-mediated amplification (TMA) at the end of combination therapy than those who were negative for it, even when they were negative for HCV RNA by conventional PCR [Gerotto et al., 2006]. Other investigators have shown that residual HCV is detectable by means of sensitive PCR in blood cells from patients who cleared HCV from the serum by IFNα and ribavirin combination therapy [Pham et al., 2004], supporting the possibility that blood cells are reservoirs of HCV replication. Taking these findings into consideration, it is conceivable that a small quantity of HCV might exist in the blood cells in some transient responders. Since direct HCV infection of monocytes or blood dendritic cells is considered to be one of the mechanisms of the functional impairment of dendritic cell [Navas et al., 2002; Goutagny et al., 2003; Ducoulombier et al., 2004], persistent HCV may delay the

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Immunotherapy of Murine Colon Cancer Using Receptor Tyrosine Kinase EphA2-derived Peptide-pulsed Dendritic Cell Vaccines

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BACKGROUND. Further optimization of dendritic cell (DC)-based vaccines is required clinically against advanced stage cancer. Given the broad range of expression levels observed in the recently defined tumor antigen EphA2 in a diverse types of cancers, especially in advanced stage or metastatic cancers, the authors evaluated the effectiveness of vaccination using DCs pulsed with EphA2-derived peptides (Eph-DCs) in a murine colon cancer model.

METHODS. EphA2 protein expression levels were evaluated in advanced colorectal carcinoma tissues from 10 patients by Western blot analysis. C57BL/6 mice were immunized with Eph-DCs twice weekly. Interferon γ (IFN-γ) ELISPOT assays were used for the analysis of CD8-positive T cells that were specific for EphA2-derived peptide. Immunized mice were challenged subcutaneously with EphA2-positive murine colorectal adenocarcinoma (MC38) mouse colon tumors or with EphA2-negative BL6 melanoma tumors. In some experiments, mice were injected with anti-CD8, anti-CD4, or antiasialo GM1 antibody to deplete corresponding lymphocyte subsets.

RESULTS. Among 10 samples of advanced colorectal carcinoma, 6 samples (60%) overexpressed EphA2. IFN-γ ELISPOT assays revealed that EphA2-derived peptide-specific CD8-positive T cells were generated by immunization with Eph-DCs. Immunization with Eph-DCs inhibited MC38 tumor growth compared with immunization using unpulsed DCs or phosphate-buffered saline. In contrast, Eph-DC vaccination had no effect on BL6 growth. Antibody depletion studies revealed that both CD8-positive T cells and CD4-positive T cells, but not natural killer cells, played critical roles in the efficacy observed for immunizations with Eph-DCs. Eph-DC vaccines resulted in long-term antitumor immunity against a rechallenge with MC38 tumor cells.

CONCLUSIONS. The current results demonstrated that Eph-DC vaccines may represent a promising preventative/therapeutic modality in the cancer setting. *Cancer* 2007;110:1469–77. © *2007 American Cancer Society.*

KEYWORDS: dendritic cells, EphA2, colorectal cancer, cancer immunotherapy.

Dendritic cell (DC)-based vaccines are attractive cancer modalities, because DCs can induce both tumor antigen-specific cytotoxic T lymphocytes (CTLs) and helper-T cells. In this regard, DCs pulsed with tumor-associated antigens in various forms, including whole cell lysates,[1] proteins,[2] peptides,[3] RNA,[4] or DNA,[5] have been proven effective in eliciting protective and therapeutic antitumor immunity in murine models. The results of several DC-based tumor vaccine trials also recently have been reported for patients who had late-stage B-cell lymphoma, melanoma, prostate cancer, and renal

cell carcinoma.[6-9] In colorectal carcinomas, DC-based vaccines using synthetic peptides derived from known tumor antigens, such as carcinoembryonic antigen (CEA), also have been reported; however, to our knowledge to date, objective clinical responses have been observed only in a minority of treated patients with colon cancer.[10-12] Thus, a new strategy for tumor antigen-derived peptide-DC vaccines is expected to improve the clinical efficacy in patients with advanced colon cancer.

The Eph family constitutes the largest family of receptor tyrosine kinases, consisting of 2 Eph classes (EphA and EphB) and 2 classes of corresponding ligands, ephrin A and ephrin B, respectively. Because they are known largely for their role in neuronal development and tissue remodeling,[13-15] it has been suggested recently that Eph receptors play a role in oncogenesis[16-18] and tumor angiogenesis.[19,20] EphA2 is overexpressed in numerous cancer types, including melanoma[21] and prostate,[8] breast,[22] lung,[23] renal cell,[24] and colorectal[25] carcinomas; and it is altered functionally to promote the development of disseminated disease in a large number of different cancers. Indeed, the highest degree of EphA2 expression among tumors is observed most commonly in metastatic lesions, suggesting that EphA2 may represent a high-priority target for immunotherapy, especially in patients with advanced stage or metastatic cancer. We previously demonstrated that some patients with renal cell carcinoma exhibited both CD8-positive and CD4-positive T-cell responses to novel, EphA2-derived epitopes and that EphA2-derived epitopes were useful for predicting disease status and outcome as immunomonitoring targets.[26,27] These results also supported the therapeutic potential of EphA2 peptide-pulsed DC (Eph-DC)-based vaccines, although they have not been evaluated to date.

In the current study, we have demonstrated that vaccination with Eph-DCs elicits EphA2-specific CTL responses that are protective against EphA2-positive tumors, but not against EphA2-negative tumors. These results support the translational development of Eph-DC vaccines for patients with EphA2-positive colon cancer.

MATERIALS AND METHODS
Mice
Female C57BL/6 mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at ages 6 weeks to 8 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Cell Lines
MC38, a mouse colon carcinoma cell derived from C57BL6/J mice, was generously provided by Dr. Kazumasa Hiroishi (Showa University School of Medicine, Tokyo, Japan). BL6, a melanoma cell line, and YAC-1, a sensitive cell line to natural killer (NK) cells, were purchased from American Type Culture Collection (ATCC) (Rockville, Md). These cell lines were maintained in complete medium (CM) (RPMI medium supplemented with 10% fetal bovine serum, 100 U/mL, penicillin and 100 μg/mL streptomycin) at 37°C in 5% carbon dioxide.

Peptides
The protein sequences of mouse EphA2 were obtained from Genbank and were analyzed for H-2K^b binding motifs using BioInformatics and Molecular Analysis Section and a proteosomal cleavage site-prediction system. The H-2K^b-binding murine (m)EphA2$_{682-689}$ epitope (VVSKYKPM) was synthesized using an automated, solid-phase peptide synthesizer in the protein synthesis facility at the University of Pittsburgh Cancer Institute and was purified (to >95%) by using reverse-phase high-performance liquid chromotography.[28]

Western Blot Analyses
The proteins in samples from 10 patients with advanced colorectal carcinoma (stage III or IV) and the lysates from mouse tumor cell lines were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were analyzed for expression of EphA2 using EphA2 monoclonal antibody (C20 Ab; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif). Blots were imaged on Hyperfilm (Amersham Bioscience, Buckinghamshire, U.K.) after using horseradish peroxidase-conjugated goat-antirabbit immunoglobulin G (Bio-Rad, Hercules, Calif) and the Super Signal West Pico Luminol Enhance Solution kit (Pierce, Rockford, Ill). Expression of β-actin served as a loading control.

Generation of DCs From Bone Marrow and DC-based Peptide Vaccines
With minor modifications, the procedure that we used in this study was described previously.[29] Briefly, C57BL/6 bone marrow cells were cultured in CM supplemented with 500 U/mL of recombinant murine granulocyte–macrophage-colony-stimulating factor (R&D Systems Inc., Minneapolis, Minn) and recombinant interleukin 4 (IL-4) (R&D Systems Inc.) for 9 days. DCs were separated by magnetic cell sorting

using CD11c microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and typically represented >90% of the harvested population of cells based on morphology and expression of the CD40, CD80, CD86, and major histocompatibility (MHC) Class II cells (data not shown). DCs were incubated with mouse Eph$_{682-689}$ peptide at a concentration of 10 μg/mL per 10^6 DC/mL CM for 2 hours at 37 °C. The cells were harvested and washed 3 times with phosphate-buffered saline (PBS) before use.[30]

IFN-γ ELISPOT Assays for Peptide-reactive CD8-positive T-cell Response

Splenocytes were harvested 5 days after subcutaneous immunization with 1×10^6 Eph-DCs twice over a 1-week interval. CD8-positive T cells were isolated selectively from splenocytes by magnetic cell sorting using CD8 microbeads (Miltenyi Biotec). Mouse IFN-γ ELISPOT assays were performed using a mouse IFN-γ ELISPOT kit (R&D Systems Inc.) according to the manufacture's instructions. IFN-γ-secreting cells appeared as blue spots. The data are represented as the mean number (±standard deviation [SD]) of IFN-γ spots per 100,000 CD8-positive T cells analyzed.

Animal Experiments

C57BL/6 mice were immunized subcutaneously into the left flank with 1×10^6 Eph-DCs or unpulsed DCs in a total volume of 100 μL of PBS twice a week. On Day 0, for the second Eph-DC immunization, 2×10^5 MC38 cells as Eph A2-positive cells or 5×10^4 BL6 cells as EphA2-negative cells were injected subcutaneously into the right flank. To assess the impact of systemic immunity from subcutaneous injection of Eph-DCs, tumor size was assessed every week and was recorded in mm^2 by determining the product of the greatest perpendicular dimensions measured by Vernier calipers. Data are reported as the average tumor area ±SD.

Cytolytic Assay

Splenocytes were harvested 14 days after tumor inoculation. Responder cells (5×10^6 per well) were restimulated in vitro with 1×10^6 MC38 cells that had been treated with 0.5 mg/mL mitomycin C (Kyowa-Hakko, Tokyo, Japan) in the presence of 30 IU/mL recombinant murine IL-2 (Strathmann Biotech, Hannover, Germany). After 5 days of in vitro restimulation, lymphocytes were subjected to 4-hour ^{51}Cr release assays against the MC38 or BL6 targets, as described previously.[29] CD4-positive and CD8-positive T cells were depleted selectively from whole splenocytes by magnetic cell sorting using CD4 and CD8 microbeads (Miltenyi Biotec), respectively. In some experiments, splenocytes were harvested 1 day after tumor inoculation and were subjected directly to 4-hour ^{51}Cr release assays against YAC-1 targets (NK cell-sensitive cells).

T-Cell and NK Cell In Vivo Depletion Experiments

To deplete T cells in vivo, anti-CD4 (GK1.5 hybridoma; ATCC) or anti-CD8 antibody (53-6.72 hybridoma; ATCC) were administered intraperitoneally 4 days and 1 day before every tumor inoculation and then every 5 days after tumor inoculation. For depletion of NK cells in vivo, we used antiasialo GM1 antibody (Wako, Osaka, Japan), which was administered intraperitoneally 1 day before tumor inoculation and then every 5 days after tumor inoculation. The efficiency of specific subset depletions was validated by flow cytometry analysis of splenocytes. In all samples, 99% of the targeted cell subset was depleted specifically (data not shown).

Tumor Rechallenge

C57BL/6 mice were immunized subcutaneously with 1×10^6 Eph-DCs twice weekly and were challenged subcutaneously with 2×10^5 MC38 cells at the second Eph-DC immunization. Forty-two days after tumor inoculation, 2×10^5 MC38 cells were injected subcutaneously into the contralateral flank of the initial MC38 tumor on Day 0. For control experiments, 2×10^5 MC38 cells were injected subcutaneously into naive C57BL/6 mice on Day 0, at the same time of MC38 rechallenge. Tumor size was assessed every week after the second tumor inoculation.

Statistical Analyses

The statistical significance of differences between the groups was determined by applying a Student t test with Welch correction or a 1-way analysis of variance after each group had been tested with equal variance and Fisher exact probability test. Statistical significance was defined as $P < .05$.

RESULTS
Expression of EphA2 in Human Colorectal Cancer Tissues and Murine Tumor Cells

We evaluated the expression of EphA2 in samples from 10 patients with colorectal carcinoma and H-2^b-syngeneic murine tumor cell lines from C57BL/6 mice by Western blot analysis using a species cross-reactive monoclonal antibody. Figure 1A shows that 6 samples (60%) of advanced-stage disease (stage III or IV) overexpressed EphA2 compared with normal colon tissues. In addition, the murine colon cancer
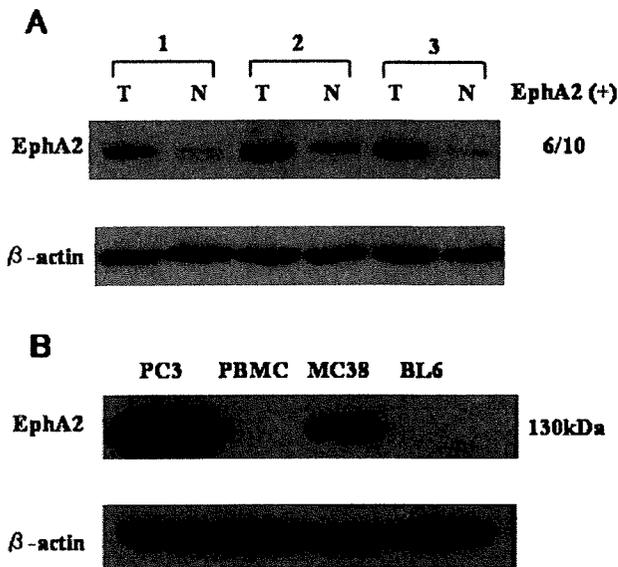
A

B

FIGURE 1. The expression levels of murine Eph receptor A2 (mEphA2) in human colorectal cancer tissues and murine tumor cells. The proteins in samples from 10 patients with colorectal carcinoma and the lysates of the murine colorectal adenocarcinoma (MC38) cell line and the BL6 melanoma cell line were evaluated for EphA2 protein expression by Western blot analysis, as described in the text. (A) Six samples (60%) of 10 advanced-stage samples (stage III or IV) expressed EphA2 protein compared with normal colon tissues. T and N indicate colon cancer tissue and its surrounding normal colon tissue, respectively. (B) The murine colon cancer cells (MC38) expressed EphA2 protein, but the murine melanoma cell line (BL6) did not. The human prostate cancer cell line PC3 was used as a positive control, and human peripheral blood mononuclear cells (PBMC) were used as a negative control. The expression of β-actin served as a loading control. KDa indicates kilodaltons.

FIGURE 2. Interferon γ (IFN-γ) ELISPOT assays for peptide-reactive, CD8-positive T cell responses. Mice were vaccinated with the indicated agent and were killed on Day 5 after immunization. CD8-positive T cells were isolated form splenocytes using CD8 microbeads and then were subjected to IFN-γ ELISPOT assays to detect Eph receptor A2 (EphA2)-derived peptide-specific cytotoxic T lymphocytes (CTLs). The data are represented as the mean number (± standard deviation) of IFN-γ spots per 100,000 CD8-positive T cells analyzed. Similar results were obtained in 3 experiments. N.S. indicates not significant; Eph-DCs, Eph peptide-pulsed dendritic cells; DCs, dendritic cells.

cell line (MC38) expressed EphA2 protein, but the murine melanoma cell line (BL6) did not (Fig. 1B).

Detection of EphA2-derived Peptide-specific CD8-positive T Cells Secreting IFN-γ After Vaccination With Eph-DCs

We performed IFN-γ ELISPOT assays to examine whether subcutaneous injection of Eph-DCs could generate CD8-positive T cells that were specific for EphA2-derived peptide in vivo. Figure 2 shows that the frequency of specific CD8-positive T cells secreting IFN-γ in mice treated with Eph-DCs was significantly higher than the frequency observed in naive mice or in mice treated with unpulsed DCs. These results demonstrate that EphA2-specific, type 1, CD8-positive T cells effectively are generated by in vivo immunization with Eph-DCs.
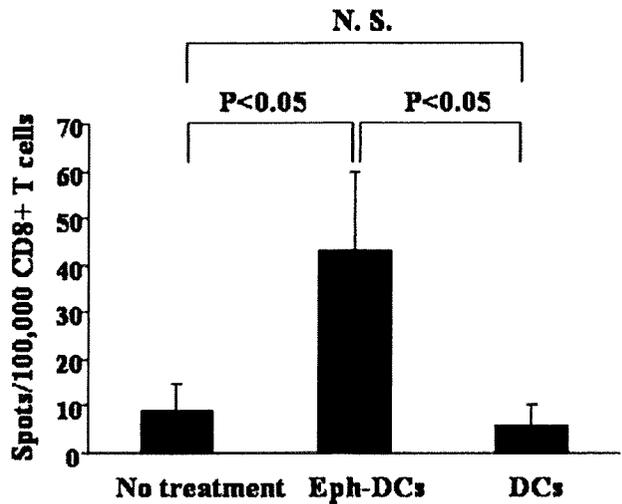
Immunization With Eph-DCs Prevents Progression of EphA2-positive Tumors, but Not EphA2-negative Tumors, In Vivo

Next, we examined whether immunization with Eph-DCs (on Days −7 and 0) would induce protective antitumor effects against EphA2-positive MC38 colon cancer. Figure 3A shows that MC38 tumor growth in mice that were immunized with Eph-DCs was inhibited strongly compared with tumor growth in mice that were immunized with unpulsed DCs ($P < .05$ on Days 21 and 28) or with PBS ($P < .05$ on Days 14, 21, and 28), whereas tumor growth in mice that were immunized with unpulsed DCs was inhibited slightly compared with tumor growth in mice that received PBS ($P < .05$ on Days 21 and 28). Conversely, in vivo growth of EphA2-negative BL6 tumors in mice that were immunized with Eph-DCs or unpulsed DCs was inhibited slightly compared with the tumor growth in mice that received PBS ($P < .05$ on Day 28). However, it is noteworthy that there was no significant difference in BL6 tumor growth between the Eph-DC group and the unpulsed DC group (Fig. 3B). These results indicate that vaccination with Eph-DCs provides specific antitumor effects against relevant EphA2-positive MC38 tumors.
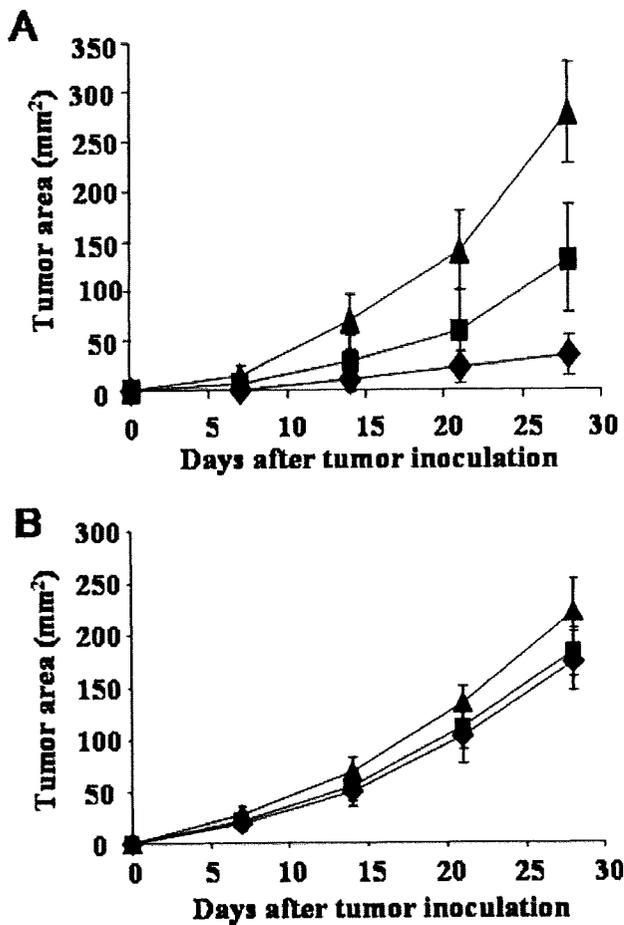
FIGURE 3. The antitumor effects of vaccination with EphA2-derived peptide-pulsed dendritic cells (Eph-DCs). C57BL/6 mice were immunized on Days −7 and 0 with 1×10^6 Eph-DCs (diamonds), unpulsed DCs (squares), or phosphate-buffered saline (PBS) (triangles). On Day 0, 2×10^5 murine colorectal adenocarcinoma (MC38) cells (A) or 5×10^4 BL6 melanoma cells (B) were injected subcutaneously. (A) MC38 tumor growth in mice that were immunized with Eph-DCs was inhibited significantly compared with tumor growth in mice treated on the other protocols ($P < .05$ on Days 14, 21, and 28 vs PBS; $P < .05$ on Days 21 and 28 vs unpulsed DCs). Tumor growth in mice that were immunized with unpulsed DCs was inhibited significantly compared with tumor growth in mice that received PBS on Days 21 and 28 ($P < .05$; N = 15 mice per group). (B) BL6 tumor growth in mice that were immunized with Eph-DCs or unpulsed DCs was inhibited compared with tumor growth in mice that received PBS ($P < .05$ on Day 28). Tumor growth in mice treated with Eph-DCs was not inhibited compared with mice treated with unpulsed DCs (N = 8 mice per group). Each data point represents the mean tumor size ±standard deviation.

Induction of Specific CTLs Against MC38 Cells After Immunization With Eph-DCs

For the next experiment, we examined whether our Eph-DC regimen could induce specific cytolytic reactivity against MC38 or BL6 cells. Splenocytes were
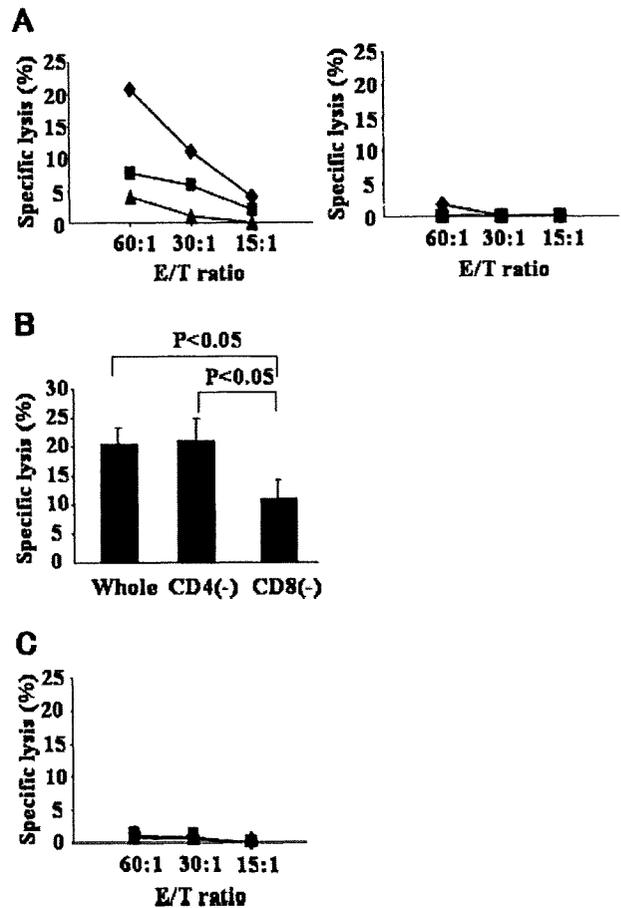
FIGURE 4. The induction of cytolytic activity by immunization with EphA2-derived peptide-pulsed dendritic cell (Eph-DC) vaccination. (A) Splenocytes were harvested from mice 14 days after final immunization with Eph-DCs (diamonds), unpulsed DCs (squares), or phosphate-buffered saline (PBS) (triangles) and tumor inoculation. Splenocytes were stimulated in vitro with mitomycin C-treated MC38 cells in the presence of recombinant human interleukin 2. After 5 days of culture, cytolytic activity was evaluated by 4-hour ^{51}Cr release cytolytic assays using MC38 cells (left) or BL6 cells (right) as targets at the indicated effector to target (E/T) ratios. (B) Before the 4-hour ^{51}Cr release cytolytic assays, whole splenocytes from Eph-DC-treated mice were depleted of CD4-positive or CD8-positive T cells by using CD4 or CD8 microbeads. Cytolytic activity against MC38 cells of depleted or nondepleted cells was compared (E/T ratio, 60:1). (C) Splenocytes were harvested 1 day after tumor inoculation. Without in vitro restimulation, whole splenocytes were subjected to 4-hour ^{51}Cr release assays against natural killer (NK)-sensitive YAC-1 cells as targets at the indicated E/T ratios. No cytolytic activity was observed against the NK-sensitive YAC-1 cells in any treatment arm (Eph-DCs [diamonds], unpulsed DCs [squares], PBS [triangles]). Similar results were obtained in 3 independent experiments (A-C).

harvested from the various treatment groups of mice that were killed 14 days after tumor inoculation. Figure 4A shows that splenocytes from mice treated with unpulsed DCs displayed weak cytolytic reactivity

against MC38 targets, whereas splenocytes from mice treated with PBS failed to exhibit detectable reactivity against this cell line. In contrast, splenocytes harvested from mice treated with Eph-DCs displayed far stronger anti-MC38 cytolytic reactivity than any control treatment group. CD8-positive, T cell-depleted splenocytes (harvested from mice treated with Eph-DCs) displayed significantly weaker anti-MC38 cytolytic reactivity than whole splenocytes; however, CD4-positive T cell-depleted splenocytes did not (Fig. 4B). Conversely, cytolytic activity was not observed against EphA2-negative cells (BL6) in any of the control/treatment arms, as shown in Figure 4A. We also harvested splenocytes 1 day after the second immunization (ie, Day 1 after tumor inoculation) to examine the potential early activation of NK cells by immunization with Eph-DCs. Figure 4C shows that no cytolytic activity was observed against the NK-sensitive YAC-1 cells in any treatment arm. These results suggest that principal antitumor effector cells in vaccinated mice are CD8-positive CTLs.

Requirement of Both CD4-positive T Cells and CD8-positive T Cells, but not NK Cells, for the Antitumor Effect of Immunization With Eph-DCs

To examine which lymphocyte subsets contributed to Eph-DC or unpulsed DC treatment, we performed depletion studies on a subset of CD4-positive T cells, CD8-positive T cells, and NK cells. Figure 5A shows that the therapeutic efficacy of Eph-DC therapy was reduced strongly in CD8-positive, T cell-depleted mice and was reduced partially in CD4-positive, T cell-depleted mice. In contrast, tumor growth still was suppressed in vaccinated mice that had been depleted of NK cells. In addition, the therapeutic efficacy of unpulsed DC therapy in CD4-positive or CD8-positive, T cell-depleted mice also was reduced, whereas that in NK cell-depleted mice was not reduced (Fig. 5B). These results suggest that both CD8-positive T cells and, to a lesser degree, CD4-positive T cells are required for the observed antitumor effects noted for Eph-DC vaccination. Both CD8-positive and CD4-positive T cells also were involved in unpulsed DC vaccination in our model.

Tumor Rechallenge

We then sought to determine whether prior Eph-DC treatment would have a durable effect on a subcutaneous rechallenge with MC38 tumor cells. C57BL/6 mice were immunized with subcutaneous injections of Eph-DCs and challenged with subcutaneous MC38 tumors. Forty-two days after the primary tumor inoculation, 2×10^5 MC38 cells were injected subcutaneously into the contralateral flank of these mice
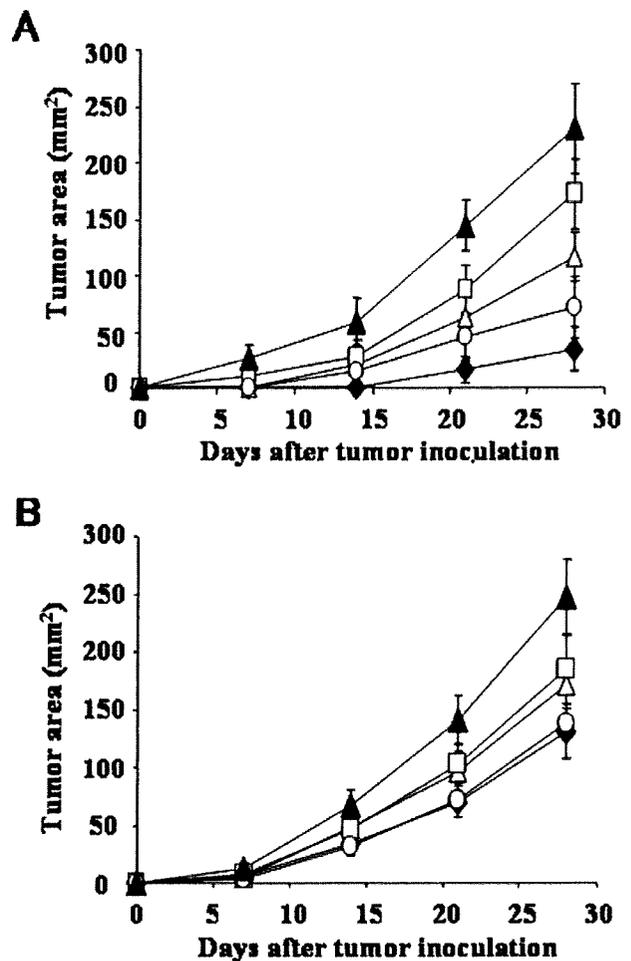

FIGURE 5. Effective Eph peptide-pulsed dendritic cell (Eph-DC) or unpulsed DC vaccination required both CD4-positive and CD8-positive T cells, but not natural killer (NK) cells. The antibody-mediated, in vivo depletion of CD4-positive T cells (open triangles), CD8-positive T cells (open squares), and NK cells (open circles) was achieved as described in the text then, and the depleted mice were treated with either Eph-DC vaccines (A) or unpulsed DC vaccines (B) on Days −7 and 0, and they received subcutaneous murine colorectal adenocarcinoma (MC38) cells (2×10^5) on Day 0. Solid triangles indicate the phosphate-buffered saline-injected group. (A) The depletion of CD8-positive T cells markedly reduced the therapeutic efficacy of Eph-DC therapy (solid diamonds) $(P < .05$ on Days 14, 21, and 28 vs Eph-DCs), and the depletion of CD4-positive T cells partially reduced its therapeutic efficacy $(P < .05$ on Days 21 and 28 vs Eph-DCs); whereas the depletion of NK cells did not reduce its therapeutic efficacy (N = 10 mice per group). (B) The depletion of CD4-positive and CD8-positive T cells also reduced the therapeutic efficacy of unpulsed DC therapy (solid diamonds) $(P < .05$ on Days 21 and 28 vs unpulsed DCs), whereas the depletion of NK cells did not reduce its therapeutic efficacy (N = 8 mice per group). Each data point represents the mean tumor size ±standard deviation.
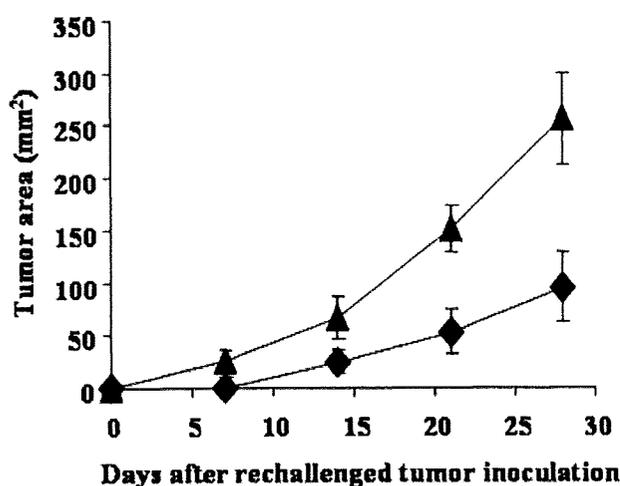
FIGURE 6. Animals treated successfully with Eph peptide-pulsed dendritic cell (Eph-DC) vaccines rejected a subsequent rechallenge with murine colorectal adenocarcinoma (MC38) (Eph receptor A2 [EphA2]-positive) tumors. Animals treated with Eph-DC vaccines prior to MC38 subcutaneous injection were rechallenged subcutaneously 42 days after the initial tumor inoculation with MC38 cells in the flank contralateral to the initial challenge. In parallel with this, naive mice were injected subcutaneously with MC38 cells as a control. Rechallenged MC38 tumors in mice that received the Eph-DC regimen (diamonds) were inhibited significantly through the chosen endpoint of these experiments on Day 28 (after MC38 rechallenge) compared with tumor growth in naive mice (triangles) ($P < .05$ on Days 14, 21, 28; N = 9 mice per group).

on Day 0. Figure 6 shows that rechallenged tumors in mice that received the Eph-DC treatment regimen were inhibited significantly in their progression through the chosen endpoint of these experiments on Day 28 ($P < .05$ vs naive mice on Days 14, 21, and 28).

DISCUSSION

Modified, DC-based vaccines using synthetic peptides derived from known tumor antigens, such as CEA, have been reported for colon cancer; although, to date, objective clinical responses have been observed only in a minority of patients who received treatment with these modalities.[10-12] This may be explained in part by the application of DC-based vaccines to immunosuppressed patients with advanced colon cancer and/or to the modest immunogenicity of tumor antigens (ie, CEA) that have been applied to date in this setting. Recently, a novel tumor antigen, EphA2, has been identified that has specific characteristics and that frequently is overexpressed in advanced cancers, suggesting that this antigen may have great potential as a target for immunotherapy, especially in

patients with advanced-stage or metastatic cancer. In the current study, 60% of colon cancer tissue samples overexpressed EphA2, consistent with a recent report by Saito et al.[25] We demonstrated that Eph-DC vaccines effectively promoted antitumor effects in a colon cancer model, suggesting that EphA2-derived CTL epitopes have the potential to serve as relevant components of novel DC-based vaccines for colon cancer.

IFN-γ ELISPOT assays revealed that immunization with Eph-DCs in normal mice resulted in the induction of specific CD8-positive T cells. Based on these results, we examined the antitumor effectiveness of Eph-DC vaccines in a syngenic, EphA2-positive MC38 colon cancer model. The Eph-DC vaccines induced antitumor effects against EphA2-positive MC38 colon carcinoma, but not against EphA2-negative BL6 melanoma, suggesting that EphA2-specific antitumor immunity was generated by Eph-DC vaccines, consistent with the results from our earlier IFN-γ ELISPOT assays.

In vitro assays revealed that the main antitumor effector cells for killing MC38 colon cancer cells were CD8-positive T cells and, possibly, CTLs. This cytolytic activity was specific for MC38 cells, because splenocytes did not kill BL6 cells. These results suggested that Eph-DC vaccines could efficiently generate specific CTLs that recognize and kill relevant EphA2-positive (but not irrelevant EphA2-negative) tumor targets.

Our in vivo lymphocyte-depletion studies demonstrated that CD8-positive T cells contributed to the inhibition of tumor growth in Eph-DC immunization and that CD4-positive T cells contributed to a lesser extent. Moreover, our tumor experimental data demonstrated that immunization with Eph-DCs maintains the antitumor effect against MC38 tumor over an extended period of time, despite the use of a single EphA2-derived CD8-positive T cell epitope in the DC-based vaccine. Typically, effector CD8-positive T cells induced by minimal CTL epitope peptides do not persist for a long time, and the induction of durable-memory CD8-positive T cells requires the support of CD4-positive T cells.[31-33] Therefore, these results suggest that Eph-DC vaccines may activate CD8-positive T cells (that recognize EphA2-derived CTL epitopes) and CD4-positive T cells (that recognize tumor antigens related to MC38 colon cancer cells), which are taken up by specifically dedicated antigen-presenting cells, and that the activated CD4-positive T cells likely contribute to the generation and maintenance of memory in EphA2-specific, CD8-positive T cells.

Immunization with control, unpulsed DCs was inhibited both EphA2-positive MC38 tumor growth

compared with PBS. Generally, unpulsed DC vaccines are not expected to generate CTLs. However, our lymphocyte-depletion studies in the MC38 tumor model demonstrated that the therapeutic efficacy of unpulsed DC therapy in CD4-positive or CD8-positive T cell-depleted mice was reduced equally. These results suggest that unpulsed DCs can induce protective antitumor effects in mice through the presentation of "self" peptides in MHC complexes to specific autoreactive CTLs, which are capable of recognizing tumor cells that also present these peptides, consistent with the previous report by Dworacki et al.[34] Moreover, unexpectedly, Eph-DC or unpulsed DC vaccines had weak antitumor effects against EphA2-negative BL6 tumors compared with PBS treatment. BL6 cells do not express EphA2, and EphA2-specific CTLs do not have cytolytic activity against BL6 tumors. Dworacki et al. reported that immunization with unpulsed DCs inhibited a variety of syngeneic tumors through the activation of both CD4-positive T cells and CD8-positive T cells,[34] suggesting that Eph-DC or unpulsed DC vaccines may activate CD4-positive and CD8-positive T cells weakly and that these cells may play a role in weakly inhibiting BL6 tumor growth.

Recent research in DC biology has revealed that DCs also contribute to innate immune responses by activating NK cells through IL-12 secretion and direct cellular interaction.[35] However, our current data demonstrated that NK cells were not involved in generating antitumor effect of Eph-DC or unpulsed DC vaccination in our lymphocyte-depletion studies. We speculate that subcutaneous, local NK cells may not be activated efficiently by immunization of Eph-DCs or unpulsed DCs, because NK cells are not so abundant in the subcutaneous tissue. Instead, if we were to apply this DC vaccine in sites rich in NK cells (ie, the liver), then this strategy may prove to be more effective in treating NK cell-sensitive liver cancers, for instance. Currently, we are evaluating these possibilities and performing histopathologic evaluations to confirm that treated animals do not exhibit autoimmune pathology in organs (such as lung, kidney, etc) that constitutively express low levels of EphA2.

Despite the recent progress and early successes reported for DC-based cancer immunotherapies, there is significant room for improvement in these regimens, especially with respect to advanced colon cancer. In this study, we demonstrated that Eph-DC vaccines revealed antitumor effects against colon cancers. In addition to CEA-based vaccines, EphA2-derived peptide-pulsed DC vaccines may represent a promising therapeutic modality against advanced colon cancers. Recently, it has been reported that some clinical trials using peptide cocktail-pulsed DCs may be useful strategies for treating patients with malignant tumors.[36,37] Therefore, DCs pulsed with multiple peptides derived from various tumor-associated antigens, including both EphA2 and CEA, may improve the therapeutic effects against advanced colon cancers.

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Signal Transducer and Activator of Transcription 3 Signaling Within Hepatocytes Attenuates Systemic Inflammatory Response and Lethality in Septic Mice

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Sepsis is an infection-induced syndrome with systemic inflammatory response leading to multi-organ failure and occasionally death. During this process, signal transducer and activator of transcription 3 (STAT3) is activated in the liver, but the significance of this molecule has not been established. We generated hepatocyte-specific STAT3-deficient mice (L-STAT3 KO) and examined the susceptibility of these mice to cecal ligation and puncture–induced peritonitis, a well-established septic model. L-STAT3 KO mice showed significantly higher mortality and produced lesser amounts of various acute phase proteins than control littermates. Although blood bacterial infection did not differ between L-STAT3 KO mice and control mice, the former showed deterioration of the systemic inflammatory response as evidenced by a significant increase in various cytokines such as tumor necrosis factor α, IFN-γ, IL-6, IL-10, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1β. A similar hyperinflammatory response was observed in another septic model caused by lipopolysaccharide (LPS) injection. In vitro analysis revealed that soluble substances derived from hepatocytes and dependent on STAT3 were critical for suppression of cytokine production from LPS-stimulated macrophage and splenocytes. Conclusion: STAT3 activation in hepatocytes can attenuate a systemic hyperinflammatory response and lethality in sepsis, in part by suppressing immune cell overactivation, implying a critical role of hepatocyte STAT3 signaling in maintaining host homeostasis. (HEPATOLOGY 2007;46: 1564-1573.)

Signal transducer and activator of transcription 3 (STAT3) mediates a signal from the IL-6 family of cytokines such as IL-6, oncostatin M, leukemia inhibitory factor, and ciliary neutrophic factor, and acti-

vates transcription of various target genes.[1] Although a STAT3 is now known to be ubiquitously expressed in variety of cells and has pleiotropic functions, it was formerly termed acute phase response factor and was first identified in the liver as an inducible DNA binding protein binding to type 2 IL-6–responsive elements within the promoter of hepatic acute phase protein (APP) genes.[2,3] Because deletion of STAT3 leads to embryonic lethality in mice, the significance of STAT3 in adult organs has been investigated using conditional knockout animals generated by the Cre/loxP recombination system.[4] Research has shown that STAT3 signaling within hepatocytes controls a variety of physiological or pathological processes, including hepatocyte proliferation after partial hepatectomy,[5] apoptosis resistance of hepatocytes during Fas-mediated liver injury,[6] and regulation of hepatic gluconeogenic genes.[7] Although STAT3 is activated in response to a rise of circulating cytokines, the significance of hepatic STAT3 has not been elucidated under systemic inflammatory conditions.

Sepsis is an infection-induced systemic syndrome, the incidence of which is estimated at 750,000 cases annually in North America with overall mortality being approxi-

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mately 30%, but rising to 40% in the elderly.[8] Sepsis develops when the initial, appropriate host response to an infection becomes amplified and then dysregulated.[9] Among those harmful or damaging responses is the rise of a variety of circulating cytokines such as IL-6, tumor necrosis factor α (TNF-α), IL-10, and IFN-γ. These cytokines lead directly to the development of systemic inflammatory response syndrome. During this process, an increasing proportion of patients will develop adult respiratory distress syndrome, disseminated intravascular coagulation, and/or acute renal failure, leading to the multiple organ dysfunction syndrome.[10] The liver is also one of the target organs of multiple organ dysfunction syndrome, although liver dysfunction may cause patient death less frequently than cardiovascular dysfunction.[11] Conversely, sepsis is a serious complication of severe liver diseases such as fulminant hepatitis[12] and decompensated cirrhosis.[13] Thus, research on the relevance of signal transduction in liver cells in the septic condition would not only satisfy basic scientific interest but would also have clinical implications.

In the present study, we used hepatocyte-specific STAT3-deficient (L-STAT3 KO) mice and examined the significance of STAT3 signaling within hepatocytes in a well-established murine model of sepsis. We found that STAT3 deficiency in hepatocytes causes exacerbation of the hyperinflammatory response by attenuating hepatic production of soluble substances that can suppress immune cell activation and also increases mortality in septic mice. This study identified an anti-inflammatory function of hepatic STAT3 signaling and its protective role against systemic inflammation, providing genetic evidence for a close link between hepatocytes and the immune system.

Materials and Methods

Animals. Mice carrying a STAT3 gene with 2 *loxP* sequences flanking exon 22 and a STAT3 null allele (*STAT3 fl/−*) have been described previously.[14] To generate mice with hepatocyte-specific STAT3 deficiency, we crossed STAT3 fl/− mice and Alb-Cre transgenic mice,[15] which express the Cre recombinase gene under the regulation of the albumin gene promoter. We crossed Alb-Cre STAT3 fl/fl mice and STAT3 fl/− mice. The resulting Alb-Cre STAT3 fl/− mice were used as L-STAT3 KO mice. Sex-matched STAT3 fl/− mice obtained from the same litter were used as control mice. All mice were used at the age of 12-15 weeks. All animals were housed under specific pathogen-free conditions and were treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Cecal Ligation and Puncture and Lipopolysaccharide Injection. Cecal ligation and puncture (CLP) is a well-established murine model of septic shock. The mice underwent CLP surgery as described previously.[16] In brief, the mice were anesthetized via intraperitoneal injection of sodium pentobarbital. Under sterile condition, the cecum was assessed via a 1-cm midline incision of the lower abdomen, ligated with a suture below the ileocecal valve, and punctured once with a 23-gauge needle. The cecum was replaced in the peritoneum, and the abdomen was closed with sutures. The mice were injected with 1 mL of lactate Ringer's solution subcutaneously for fluid resuscitation. As another septic model, lipopolysaccharide (LPS) (form *Escherichia coli* O55: B5; Sigma, St. Louis, MO) was injected intraperitoneally at a dose of 4 mg/kg body weight.

Preparation of Peritoneal Macrophage. To isolate peritoneal macrophages, we injected mice intraperitoneally with 2 mL of 4% thioglycollate. Peritoneal exudates cells were isolated from the peritoneal cavity 4 days after injection. The cells were incubated for 4 hours in 96-well plates and washed 3 times with phosphate-buffered saline. We used the adherent cells as peritoneal macrophages for further experiments.

Determination of the Bacterial Load. Mice were sacrificed 24 hours after CLP surgery. Samples of blood were obtained in sterile condition. Fifty microliters of the blood were then plated on heart-infusion plates. The heart-infusion plates were incubated at 37°C overnight, and the number of bacteria colonies was counted. Results were expressed as $\log_{10}$ of CFU.

Blood Biochemistry. Blood samples were obtained 24 hours after CLP or LPS injection. Acute phase proteins, cytokines, and chemokines in plasma were determined via MultiAnalyte Profile testing (Rules Based Medicine, Austin, TX). Levels of serum ALT and creatinine were measured with a standard UV method using a Hitachi type 7170 automatic analyzer (Tokyo, Japan).

Measurement of Culture Supernatant. Levels of cytokines (TNF-α, IL-6, IL-10, and IFN-γ) in the culture supernatants were measured using commercially available ELISA kits in accordance with the manufacturer's instructions (BD Biosciences-Pharmingen, San Diego, CA). Haptoglobin was determined in cell-free supernatants by using a commercially available ELISA kit (Immunology Constants Laboratory, Newberg, OR).

Western Blot Analysis. The total cellular protein was extracted with the RIPA buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, and 50 mM sodium fluoride in phosphate-buffered saline (pH 7.4). Twenty micrograms of protein were separated

via 7.5% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline 0.1% Tween 20 containing 5% skim milk or Blocking One-P (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, the membrane was incubated overnight at 4°C with antibodies to STAT3 or tyrosine[705]-phosphorylated STAT3 (Cell Signaling Technology, Danvers, MA), respectively. After washing with Tris-buffered saline 0.1% Tween 20, the membrane was incubated with anti–horseradish peroxidase–linked antibody for 1 hour at room temperature. The immune complex was detected by an enhanced chemiluminescent assay. In some experiments, tyrosine[701]-phosphorylated STAT1 antibody (Cell Signaling Technology) was also used. This antibody recognizes the phosphorylated form of both STAT1α and STAT1β.

Histology and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling. The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed using an ApopTag kit according to the manufacturer's instructions (Serological Corporation, Norcross, GA).

Primary Culture of Hepatocytes. Livers were digested using a standard in situ 2-step collagenase perfusion procedure (Gibco BRL, Rockville, MD). Hepatocytes were isolated from nonparenchymal cells via subsequent centrifugation at 50g for 1 minute. In a selected experiment, nonparenchymal cells in the supernatants were pelleted at 1,500 rpm for 5 minutes and subjected to western blot analysis. Isolated hepatocytes with >90% viability were cultured in Williams' medium E containing 10% fetal bovine serum overnight. On the next day, the cells were stimulated with recombinant IL-6 (PeproTech, London, UK). The cells were harvested after 2 hours for the analysis of STAT3 activation. In another experiment, supernatants were harvested after 48 hours.

Cytokine Production by Macrophage and Splenocytes. The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA). RAW cells were plated at a density of 5 × 10^{5}/well in a 96-well plate and were incubated at 37°C in culture supernatants of hepatocyte from L-STAT3 KO mice or control mice. As a control, RAW cells were also cultured in Williams' medium E. After 24 hours, LPS was added to achieve a final concentration of 100 ng/mL. After 24 hours of incubation at 37°C in an atmosphere of 5% CO_2, the supernatant was collected and stored at −80°C for measurement of TNF-α, IL-6, and IL-10. Splenocytes were isolated by way of a standard

procedure for wild-type mice[17] and incubated with hepatocyte culture supernatant. Twenty-four hours after incubation, the cells were stimulated with LPS (1,000 ng/mL) for 24 hours. The resultant culture supernatant was subjected to IFN-γ ELISA.

Statistics. Kaplan-Meier curves were used to show survival over time. Data are expressed as interquartile range and median and compared using the Mann-Whitney U test. Statistical significance was set at $P < 0.05$.

Results

Mice with hepatocyte-specific STAT3 deficiency were produced by crossing floxed STAT3 mice and Alb-Cre transgenic mice carrying the Cre recombinase gene under the regulation of the albumin gene promoter. L-STAT3 KO mice were born and grew without any gross abnormality. Western blot analysis revealed that STAT3 expression was substantially decreased in the liver but not in other organs (Fig. 1A). Isolation of hepatocytes from nonparenchymal cells by liver perfusion followed by centrifugation confirmed that STAT3 deficiency is specific in hepatocytes (Fig. 1B). In addition, STAT3 expression did not differ in peritoneal macrophages between L-STAT3 KO mice and control littermates (Fig. 1C). Those cells isolated from L-STAT3 KO mice produced similar levels of TNF-α in response to LPS compared with those from control littermates (Fig. 1D).

L-STAT3 KO Mice Are More Vulnerable to Septic Shock. To examine the role of hepatic STAT3 during septic shock, we used a well-examined clinically relevant murine model of sepsis performed by CLP.[16] CLP clearly activated liver STAT3, which was determined via phosphorylation of STAT3 in control mice (Fig. 2A), in agreement with a previous report.[18] Liver STAT3 activation during sepsis is mostly due to the activation of STAT3 in hepatocytes, because liver STAT3 was only marginally activated in L-STAT3 KO mice. CLP activated liver STAT1 both in L-STAT3 KO mice and wild-type mice, suggesting that the absence of STAT3 does not affect the activation of other STATs. Given that STAT3 is a well-known mediator for APP,[19] we measured APPs such as fibrinogen and haptoglobin in plasma after CLP (Fig. 2B). The levels of fibrinogen and haptoglobin clearly increased after CLP in wild-type mice. In contrast, induction of fibrinogen was completely diminished in L-STAT3 KO mice, whereas that of haptoglobin was partially inhibited. This is consistent with the previous notion that fibrinogen is a class 2 gene and haptoglobin is a class 1 gene; the class 2 gene is predominantly regulated by type 2 IL-6 responsive elements binding to STAT[20] and the class 1 gene by both type 1 IL-6 responsive elements