cynomolgus macaques. Busulfan was orally administered to the macaques twice at 10 mg/kg each (CD4T-1 and CD4T-2) or 6 mg/kg each (CD4T-3) [38]. The expanded cells were harvested, washed three times with PBS, and re-suspended in PBS containing 10% autologous plasma. The collected cells were infused intravenously to monkeys at the speed of 1 ml per minute.

Flow cytometry analysis

The cell surface markers of the expanded cells and peripheral blood mononuclear cells (PBMC) were analyzed using FACSCalibur (BD Bioscience) and FACSCanto (BD Bioscience), and data analysis was performed using CellQuest software (BD Bioscience), FACSDiva software (BD Bioscience) or FlowJo software (Tree Star, Inc., Ashland, OR). The following antibodies were used for staining: anti-CD3 (SP34-2, PerCP), anti-CD4 (L200, FITC), anti-CD25 (2A3, FITC), anti-CD28 (CD28.2, PE), anti-CD95 (DX2, FITC), anti-CXCR4 (12G5, PE) and anti-integrin-β7 (FIB504, PE), which were obtained from BD Bioscience. The anti-CD49d (HP2/1, FITC) antibody was obtained from Beckman Coulter (Fullerton, CA), and the anti-CD271 (LNGFR, PE and APC) antibodies were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Measurement of hematological data

Two ml of blood was prepared every week. Blood samples were used to measure the white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit values, mean corpuscular volume, mean cell hemoglobin concentration and platelet (PLT) count using a Sysmex K-4500 instrument (Toaiyouddenshi, Kobe, Japan). The concentrations of the biochemical markers in blood samples were also monitored including total proteins, albumin, blood urea nitrogen, glucose, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase, creatine phosphokinase, lactate dehydrogenase, creatine, sodium, potassium, chlorine and C-reactive protein using an AU400 instrument (Olympus Medical Systems, Tokyo, Japan).

Quantification of gene-modified CD4+ T cells

The existence and persistence of genetically modified CD4+ T cells were monitored by measuring the proviral genome of the transgene using quantitative real-time PCR. DNA samples were extracted from 2×10^6 PBMCs using a Gentra Puregene Blood Kit (QIAGEN, Hilden, Germany). The proviral copy number of the transgene was calculated from 400 ng of genomic DNA with quantitative PCR using a Cycleave RT-PCR Core Kit (Takara Bio) and Provirus Copy Number Detection Primer Set (Takara Bio) according to the manufacturer's instructions. The reaction was performed with the Thermal Cycler Dice Real Time System (Takara Bio), and the data was analyzed using Multiplate RQ software (Takara Bio). For each run, a standard curve was generated from the pMT-MFR-PL2 plasmid, whose copy numbers were already known. Based on the standard curve, the amount of infused cells was quantified.

Detection of anti-MazF antibodies in macaque blood after transplantation of MazF-Tmac cells

To examine whether anti-MazF antibodies can be generated after the transplantation of MazF-Tmac cells, the plasma isolated from the macaques was analyzed. In order to detect anti-MazF antibodies, purified MazF protein or anti-monkey IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) was precoated onto the wells of a 96-well microplate and subsequently blocked with PBS-1% BSA. The plasma samples were isolated from the CD4T-2 at day 0, 33, 75, and 103 after transplantation and were diluted to 500,000-fold, 50,000-fold, and 10,000-fold. Cynomolgus macaque IgG purified from normal macaque plasma with Melon Gel IgG purification Kit (Thermo Fisher Scientific, Rockford, IL, USA) was used as a control for this reaction. The two-fold serial dilutions of the IgG (1 ng/ml to 64 ng/ml) and the diluted plasma samples, as described above, were separately added to each well. After an overnight incubation at 4°C, the wells were washed with PBS-1% BSA. The POD-conjugated anti-monkey IgG (Nordic Immunological Laboratories) was then added to the wells. After 4 hours of incubation at room temperature, the wells were washed three times with PBS-1% BSA followed by the addition of the substrate solution (o-Phenylenediamine, Sigma). The optical density of each well was read at 490/650 nm using a 680XR microplate reader (Bio-Rad Laboratories, Hercules, CA) after stopping the reaction with H_2SO_4 stop solution (Figure S1).

Examination of the anti-viral efficacy of MazF-Tmac cells harvested from a monkey

To examine the function of the *mazF* gene in cells harvested from a MazF-Tmac-transplanted monkey, the frozen lymphoid cells from CD4T-1 at autopsy (214 days post-infusion of MazF-Tmac cells) were recovered, CD4+ T cells were selected using a CD4+ T Cell Isolation Kit (Miltenyi Biotec), stimulated with anti-CD3/CD28 beads at a cell-to-bead ration of 1:1, and expanded in GT-T503 medium supplemented with 10% FBS, 200 IU recombinant human interleukin-2, 2 mM L-glutamine, 2.5 μg/ml Fungizone, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 7 days of expansion, the genetically modified cells expressing ΔLNGFR+ were concentrated with an anti-CD271 monoclonal antibody (CD271 MicroBeads, Miltenyi Biotec) and expanded for 4 days. The cells from the CD271-negative fraction were also harvested and expanded as control non-gene modified CD4+ T cells. The expanded CD271-enriched cells and CD271-negative cells were infected with SHIV 89.6P at the MOI of 0.01 and cultured for 6 more days. Culture supernatants and cell pellets were harvested at 6 days post-infection. RNA in the culture supernatant was recovered with the QIAamp Viral RNA Mini Kit (QIAGEN) and SHIV RNA levels in the culture supernatant were determined by quantitative real-time PCR with a set of specific primers specific for the SHIV *gag* region [39]. In order to detect the Tat-dependent expression of MazF in the CD271-enriched MazF-Tmac cells harvested from the monkey, the cells were transduced with the Tat expression retroviral vector M-LTR-Tat-ZG [6] in the presence of RetroNectin® as per the manufacturer's instruction. Twenty hours after Tat transduction, the cells were harvested, counted by trypan blue exclusion assay, washed twice with PBS, and 5×10^5 cells were suspended in 50 μl of 1× SDS sample buffer. The cell samples were incubated at 95°C for 10 min, and 5 μl of each cell sample was used for western blot analysis. For gel electrophoresis of proteins, the sample solutions described above were loaded into the wells of a 4–20% Tris-Glycine gel (Atto, Tokyo, Japan). After completion of electrophoresis, the gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) with papers containing transfer buffer using the semi-dry method at 60 mA (constant voltage) for 60 min. The membrane was cut in half horizontally around the 20 kDa protein band of the pre-stained protein marker (Bio-Rad Laboratories). The upper part of the membrane was used to detect the α-tubulin (50 kDa) as an internal standard, while the lower part of the membrane was used to detect MazF (12 kDa). After blocking, the membranes were then incubated overnight at 4°C in the blocking buffer (5% skim milk in PBS)

containing 1 μg/ml anti-α-tublin antibody (Cell Signaling Technology) and 1 μg/ml anti-MazF polyclonal antibody (rabbit, in-house preparation), respectively. Each membrane was washed three times and subsequently incubated at room temperature for 1 hour in 10 ml of the blocking buffer containing the 10,000-fold diluted goat anti-IgG rabbit antibody (peroxidase conjugated, Thermo Fisher Scientific). The membrane was washed five times by gentle shaking in the washing buffer at room temperature for 5 min. The membrane was soaked at room temperature for 5 min in substrate solution (SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate, Thermo Scientific). Protein signals were detected by a CCD camera (LuminoShot 400 Jr, Takara Bio), which captures a digital image of the western blot.

Collection of lymphocyte from several organs

Several organs were collected following euthanasia of the monkeys. After thoracotomy, the right atrium was incised, and 2 L of heparinized PBS was infused into the left ventricle using an 18-gauge needle. After perfusion, several organs were collected, and lymphocytes were separated using the following method: samples of spleen, thymus, liver, bone marrow, and axillary, inguinal and mesenteric LNs were minced and filtered through a 40 μm nylon filter (BD Bioscience); lymphocyte of the small intestine were collected by the Percoll (GE Healthcare, Castle Hill, Australia) density-gradient centrifugation method as described previously [39]; and lymphocytes obtained from each organ were used for the flow cytometric analysis, and extracted DNA was used for quantification PCR.

In vivo homing analysis

CD4T-4 was used for homing analysis. Isolated CD4+ T cells were stimulated with anti-CD3/CD28 beads and cultured in GT-T503 medium supplemented with 10% FBS, 200 IU IL-2, 2 mM L-glutamine, and 2.5 μg/ml Fungizone. After 4 days of expansion, activated CD4+ T cells were divided into two culture bags (ClutiLife Eva), and 10 nM retinoic acid (Sigma) was added to one of the bags. After an additional 5 days of incubation, expanded cells with or without retinoic acid were harvested and labeled with 2 mM PKH26 (Sigma) or 5 μM CFSE (Sigma), respectively, according to the manufacturer's instructions. Thereafter, the cells were washed three times with PBS, mixed in PBS containing 10% autologous plasma and infused into the macaque. Then, CD4T-4 was euthanized at 3 days after transplantation. Lymphocytes from several organs were collected as previously described, and the distributions of labeled lymphocytes were detected by flow cytometric analysis. The specimens from several organs were fixed in buffered formaldehyde and embedded in plastic. Serial sections were made using a diamond saw. The slides were then analyzed under a fluorescence microscope to detect the distribution of the expanded cells in the distal organ specimens.

Supporting Information

Figure S1 Raw data of 490/650 nm absorbance. The raw data of the optical density of each well at 490/650 nm was read using a microplate reader 680XR (Bio-Rad Laboratories, Hercules, CA) is represented.
(PDF)

Figure S2 Photographs of histopathological analysis. Individual photographic data of histopathological analysis of CD4T-1, -2, and -3 in Table 3 is represented.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HC NS YY KT JM IK. Performed the experiments: HC NS HT HS NA. Analyzed the data: HC NS HS NA. Contributed reagents/materials/analysis tools: NS HT HS NA. Wrote the paper: HC NS.

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HTLV-I-Infected Breast Milk Macrophages Inhibit Monocyte Differentiation to Dendritic Cells

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Abstract

Human T-cell leukemia virus type I (HTLV-I), a causative agent of adult T-cell leukemia (ATL), is transmitted from mother to child, predominantly by breastfeeding. Oral HTLV-I infection and infection early in life are associated with a subsequent risk of ATL. Although the pathogenic mechanisms of ATL remain largely unknown, the host immune system seems to play an important role in HTLV-I pathogenesis. Previous studies have shown that monocytes from ATL patients had reduced capacity for dendritic cell (DC) differentiation. Therefore, we performed the present study to clarify the mechanisms responsible for the impairment of DC differentiation using HTLV-I-infected breast milk macrophages (HTLV-BrMMø). We found that when $CD14^+$ monocytes were cultured with GM-CSF and IL-4 in the presence of HTLV-BrMMø, they altered the surface phenotype of immature DCs and the stimulatory capacity of T-cell proliferation. The presence of HTLV-BrMMø significantly blocked the increased expression of CD1a, CD1b, CD11b, DC-SIGN, and HLA-DR; however, increased expression of CD1d and CD86 was observed. These effects could be partially replicated by incubation with culture supernatants from HTLV-BrMMø. The impairment of monocyte differentiation might be not due to HTLV-I infection of monocytes, but might be due to unknown soluble factors. Since other HTLV-I-infected cells exhibited similar inhibitory effects on monocyte differentiation to DCs, we speculated that HTLV-I infection might cause the production of some inhibitory cytokines in infected cells. Identifying the factors responsible for the impairment of monocyte differentiation to DCs may be helpful to understand HTLV-I pathogenesis.

Introduction

Human T-cell leukemia virus type I (HTLV-I) is the first oncoretrovirus to be discovered in humans (31). Worldwide, the infected population is estimated to be about 10–20 million individuals. Approximately 90% of infected individuals remain asymptomatic carriers during their lives. It is estimated that 2–5% of infected individuals develop adult T-cell leukemia/lymphoma (ATL) (3); further, another small proportion (0.1–2%) will develop a clinically distinct progressive neurological disease known as HTLV-I-associated myelopathy (HAM) or tropical spastic paraparesis (TSP) (28,30).

Although the pathogenic mechanisms of ATL remain largely unclear, HTLV-I-encoded gene products such as Tax or basic leucine zipper factor (HBZ) may be among the key factors ultimately leading to the development of ATL (23). Moreover, the host immune system also seems to play an important role in HTLV-I pathogenesis. It has been shown that HTLV-I-specific T-cell responses are critical for a low viral load to be maintained (7,20). However, HTLV-I-specific cytotoxic T cells (CTLs) can be induced from peripheral blood mononuclear cells (PBMCs) of asymptomatic carriers, but rarely from those of ATL patients or those who fail to respond to *ex vivo* stimulation (4). Clarifying the reasons why HTLV-I-specific immune responses are not effective in HTLV-I-infected patients is critical to prevent the development of ATL.

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) involved in the capture and processing of antigens and are capable of inducing primary responses of T cells. Therefore, the modifications of DCs during their differentiation process have a direct effect on T-cell responses. Previous studies have shown that monocytes from ATL or HAM/TSP patients had a reduced capacity for DC differentiation *in vitro* (1,22,29), and the numbers of myeloid DC (myDC) and plasmacytoid DC (pcDC) cells were decreased in ATL patients (15). HTLV-I can efficiently infect monocytes and DCs as well as T cells (19); however, Azakami *et al.* have reported that there was no close correlation between a high proviral load of PBMCs and the capacity for DC

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differentiation (5). Furthermore, monocytes that exhibited impaired differentiation to DCs were not always positive for HTLV-I (29). These findings suggest that impaired differentiation of DCs might be induced under infectious disease conditions rather than the infected state of monocytes.

HTLV-I is transmitted primarily in three ways: mother to child (11), sexual transmission (40), and via blood transfusion (38). Among these routes, mother-to-child transmission through breast milk is the most important route to stop, because oral HTLV-I infection and infection early in life are associated with a subsequent risk of ATL (11,14). Previously, we demonstrated that $CD14^+$ breast milk macrophages (BrMMø), the majority of cells in breast milk, particularly in early colostrum, were capable of producing granulocyte macrophage-colony stimulating factor (GM-CSF) spontaneously, and differentiating into $CD1^+$ DCs by stimulation with exogenously added interleukin-4 (IL-4) (16,32,39). Moreover, they could differentiate into DC-like cells by HTLV-I infection (36). An appropriate number of HTLV-I-infected BrMMø efficiently induced T-cell proliferation and transmitted HTLV-I virions to activated T cells (36); that is, HTLV-I-infected BrMMø might serve as a reservoir as well as a transmitter of HTLV-I. Recently, we observed that when $CD14^+$ monocytes were cultured with GM-CSF and IL-4 in the presence of HTLV-I-infected BrMMø, they altered the surface phenotype of immature DCs and the stimulatory capacity of T-cell proliferation. This inhibitory effect could be partially replicated by incubation with culture supernatants from HTLV-I-infected BrMMø. In the present study, considering the possibility that HTLV-I-infected BrMMø might modulate the immune systems in newborns through breastfeeding, we examined how HTLV-I-infected BrMMø affect the process of monocyte differentiation to DCs.

Materials and Methods

Preparation of cells

Fresh BrMMø were isolated from breast milk by Lymphosepar I (Immuno-Biological Laboratories, Gunma, Japan) gradient centrifugation, followed by adherence to polystyrene tissue culture dishes for 1 h at 37°C. Adherent cells were then removed by incubation with 5 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 4°C and cultured in T-cell culture medium (CTM) (35), composed of RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L non-essential amino acids, a mixture of vitamins, 1 mmol/L HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 mmol/L 2-mercaptoethanol, and heat-inactivated 10% fetal calf serum. PBMCs were isolated from healthy donors using Lymphosepar I gradient centrifugation. $CD14^+$ monocytes were separated from PBMCs by a human monocyte enrichment kit (Stemcell Technology, Vancouver, B.C., Canada), routinely resulting in >90% purity of $CD14^+$ cells. Cells were cultured in CTM supplemented with 200 ng/mL GM-CSF and 10 ng/mL IL-4 to obtain DCs. HTLV-I-producing cell lines of BrMMø (HTLV-BrMMø) and DC (HTLV-DC) were established as described previously (36). Briefly, BrMMø or immature DCs were infected with 0.5 mL supernatant containing 354 ng/mL HTLV-I p19 from MT-2 (26), an HTLV-I-producing T-cell line, for 18 h at 37°C, and further propagated with CTM. B95-a cells [an Epstein-Barr virus-transformed marmoset B cell line (21)], MOLT-4 cells [human acute lymphoblastic leukemia cell line (25)], and MT-2 cells were cultured in CTM. This study was approved by the Review Board of Nippon Medical School and all human participants gave written informed consent.

Co-culture experiments

$CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 either in the presence or absence of the following irradiated (30 Gy) test cells: HTLV-BrMMø (36), MT-2 (26), HTLV-DC, B95-a (21), and MOLT-4 (25), at a 30:1 monocyte:test cell ratio for 3 d. Test cells were labeled with 2×10^{-6} M PKH67 (green fluorescence) or PKH26 (red fluorescence) (Sigma-Aldrich, St. Louis, MO) to differentiate them from monocytes. Titration of HTLV-BrMMø to monocyte cell ratio of 1:10 up to 1:90 was performed using different numbers of HTLV-BrMMø to examine whether the inhibitory effect of HTLV-BrMMø on monocyte differentiation occurs in a dose-dependent manner. To obtain culture supernatants from various cell lines, 3×10^4 cells/mL of each cell line were cultured in CTM for 7 d and the supernatants were collected after centrifugation at 500 g for 10 min. $CD14^+$ monocytes were cultured with GM-CSF and IL-4 either in the presence or absence of culture supernatants for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4 for 2 d. Purified HTLV-I virions were prepared from culture supernatants from HTLV-BrMMø (HTLV-BrMMø-sup) using the Fast-Trap lentivirus purification and concentration kit (Millipore, Billerica, MA) according to the manufacturer's instructions. To examine the characteristics of the active substances in HTLV-BrMMø-sup, we heated HTLV-BrMMø-sup to 90°C for 30 min, and subjected HTLV-BrMMø-sup to treatment with 1500 U/mL trypsin (Sigma-Aldrich) at 37°C for 3 h. To determine the molecular weight of the active substance in HTLV-BrMMø-sup, they were fractionated by Amicon Ultra-15 (Millipore).

Antibodies and flow cytometric analysis

Cells were pelleted and re-suspended at a concentration of $<5\times10^5$ cells in 50 mL PBS with 0.1% NaN_3 and 1% human serum containing each monoclonal antibody. For flow cytometric analysis, phycoerythrin (PE)-conjugated antibodies to CD1a, CD1d, CD11b, CD14, CD83, and CD86, as well as fluorescein isothiocyanate (FITC)-conjugated antibody to HLA-DR were purchased from BD Bioscience (Franklin Lakes, NJ). PE-conjugated antibodies to CD1b and CD1c, and allophycocyanin (APC)-conjugated antibody to CD3, were from BioLegend (San Diego, CA). PE-conjugated antibodies to CD40 and DC-SIGN were from eBioscience Inc. (San Diego, CA) and R&D Systems (Minneapolis, MN), respectively. FITC-conjugated antibody to Vα24 was from Immunotech (Marseille, France). After 30 min incubation with each antibody at 4°C, the cells were washed and resuspended in PBS for analysis by FACScan (BD Bioscience) and Flow Jo analysis software (Tree Star Inc., Ashland, OR). Neutralizing antibodies against IL-10 were purchased from Miltenyi Biotec Inc. (Auburn, CA).

Detection of HTLV-I proviral DNA

$CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence of HTLV-BrMMø-sup

for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4 for 3 d. DNA was isolated from monocytes using Blood & Tissue Genome Mini kits (Viogene, Sunnyvale, CA). HTLV-I proviral DNA was detected by polymerase chain reaction (PCR) using the following primers for the Tax gene to generate a 244-bp fragment: 5′-TGTTTGGAGACTGTGTACAAGGCG-3′ and 5′-GTTGTATGATTGGCGGGGTAA-3′.

Mixed lymphocyte reaction (MLR) assays

For assessment of T-cell stimulation in allogeneic MLR, both PBMCs and $CD14^+$ monocytes were cultured with HTLV-BrMMø-sup for 18 h. After washing with PBS, cells were further incubated in CTM supplemented with GM-CSF and IL-4 for 2 d. The cells were then irradiated (30 Gy) and used as APCs. Purified allogeneic T cells (5×10^4 cells/well) were cultured with the indicated number of APCs in 96-well U-bottomed tissue culture plates at 37°C for 5 d. Proliferation was measured by the addition of 0.5 μCi/well ^{3}H-thymidine/well for the last 16 h, and the plates were harvested and counted using a β-counter (1450 Microbeta Trilux; Wallac, Gaithersburg, MD).

Measurement of virus and cytokine production

IL-10 and IL-12 concentrations were measured using the DuoSet ELISA Development Kit (R&D Systems) according to the manufacturer's instructions. HTLV-I p19 core antigens were measured using the HTLV p19 Antigen ELISA Kit (ZeptoMetrx Corporation, Buffalo, NY).

Induction of iNKT cells

PBMCs were cultured with 10 ng/mL α-galactosylceramide (α-GalCer; Enzo Life Sciences Inc., Farmingdale, NY) either in the presence or the absence of HTLV-BrMMø-sup for 7 d. Cell cultures were fed by replacing a half-volume of media every other day with fresh CTM with IL-2 (10 U/mL). No additional α-GalCer was added during cell feeding.

Statistical analysis

Student's *t*-test was used to determine the significance of differences in means. A p value of <0.05 was considered significant.

Results

HTLV-BrMMø and supernatants from HTLV-BrMMø inhibit monocyte differentiation to immature DCs

In order to study whether HTLV-BrMMø modulates DC function by regulating the expression of surface molecules, $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence or absence of HTLV-BrMMø for 3 d. Monocytes were characterized by side scatter and forward scatter profiles, followed by gating on PKH-negative cells by FACS analysis. Generally, when $CD14^+$ monocytes are stimulated with GM-CSF and IL-4, they differentiate into immature DCs, displaying increased expression of CD1a, in contrast to decreased or absent CD14 expression. The presence of HTLV-BrMMø significantly blocked the increased expression of CD1a, CD1b, CD11b, DC-SIGN, and HLA-DR, and slightly blocked that of CD1c. Interestingly, increased expression of CD1d and CD86 was observed. The expression levels of CD14 and CD40 were not altered (Fig 1A). This inhibitory effect was not found in co-cultures with uninfected BrMMø (Fig. 1B). When $CD14^+$ monocytes and HTLV-BrMMø were separated by a permeable filter, monocytes failed to acquire CD1a expression (data not shown). These findings suggest that HTLV-BrMMø are capable of modulating the expression of surface molecules on monocytes by secreting cytokines; therefore, we next examined the effect of culture supernatants from HTLV-BrMMø (HTLV-BrMMø-sup) on monocyte differentiation to DCs. $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence or absence of HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4. Flow cytometric analysis performed on day 3 of culture revealed a significantly decreased expression of CD1a, DC-SIGN and HLA-DR, and increased expression of CD1d and CD86, as observed in co-cultures with HTLV-BrMMø; however, the expression levels of CD1b, CD1c, and CD11b were not altered (Fig. 1C). This inhibitory effect was not found in culture supernatants from uninfected BrMMø (Fig. 1D). These data implied that HTLV-BrMMø were capable of modulating the expression of surface molecules on monocytes, mainly through cell–cell interactions, and partly by secreting cytokines. HTLV-BrMMø-sup induced the dose-dependent inhibition of CD1a expression, and complete loss of CD1a expression resulted after more than 18 h of incubation with HTLV-BrMMø-sup (Fig. 1E).

◀F1

Characterization of soluble factors responsible for the impairment of monocyte differentiation

Culture supernatants of monocytes with HTLV-BrMMø contained less than 10 ng/mL HTLV-I p19 (Fig 2A), whereas HTLV-BrMMø-sup contained more than 50 ng/mL HTLV-I-p19. HTLV-I can infect monocytes and DCs as well as T cells. Thus, to explore whether the impairment of monocyte differentiation to DCs could be caused by HTLV-I infection of monocytes, we measured HTLV-I proviral load in monocytes by PCR. Although monocytes incubated with HTLV-BrMMø-sup exhibited downregulation of CD1a, genomic DNA from these monocytes did not contain HTLV-I proviral DNA (Fig. 2B). To further examine whether HTLV-I virions were required for the impairment of monocyte differentiation to DCs, experiments were performed in which excessive amounts of purified HTLV-I virions were added to $CD14^+$ monocytes cultured with GM-CSF and IL-4. One μg/mL of purified HTLV-I virions did not cause a loss of CD1a expression (Fig. 2C). Furthermore, when HTLV-BrMMø-sup was treated with ultraviolet irradiation or heating, and the expression levels of CD1a on monocytes remained down-regulated. These findings suggest that the impairment of monocyte differentiation does not necessarily require HTLV-I infection of monocytes.

◀F2

IL-10 is a potent immunosuppressive cytokine that reduces the expression of MHC class II, CD80, and CD86 on DCs, and inhibits the production of proinflammatory cytokines by DCs (2). Monocytes and macrophages are known to produce IL-10 by various stimuli, and Makino *et al.* have reported that HTLV-I-infected monocytes secreted a large amount of IL-10 (22). Co-cultures of

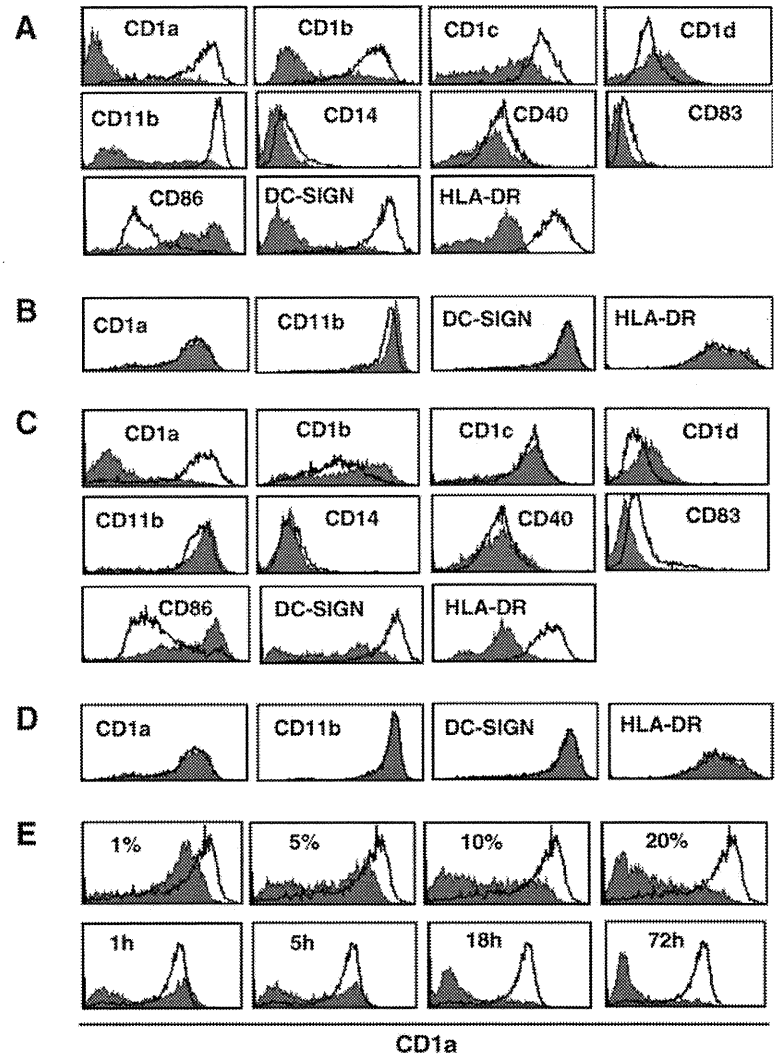

FIG. 1. Effect of HTLV-BrMMø and culture supernatants from HTLV-BrMMø on monocyte differentiation. $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4, (**A**) either in the presence (filled histograms) or absence (open histograms) of PKH-labeled HTLV-BrMMø at a 30:1 monocyte:HTLV-BrMMø ratio for 3 d. Viable cells were gated from nonviable cells by their distinct forward scatter versus side scatter, and phenotype analysis of monocytes was performed by gating on PKH-negative cells, (**B**) either in the presence (filled histograms) or absence (open histograms) of PKH-labeled uninfected BrMMø at a 30:1 monocyte:HTLV-BrMMø ratio for 3 d. (**C**) Also, either in the presence or absence of HTLV-BrMMø-sup at a final concentration of 20% for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4. Flow cytometric analysis was performed on day 3, (**D**) either in the presence (filled histograms) or absence (open histograms) of culture supernatants from uninfected BrMMø at a final concentration of 20% for 3 d. (**E**) Top row: Also, either in the presence or absence of HTLV-BrMMø-sup at a final concentration of 1–20% for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4. Bottom row: Also, either in the presence or absence of HTLV-BrMMø-sup at a final concentration of 20% for 1–72 h. Flow cytometric analysis was performed on day 3.

monocytes with irradiated HTLV-BrMMø or HTLV-DC produced less IL-10 than co-cultures of monocytes with irradiated MT-2 for 3 d (Fig. 2D, upper panels). We then measured IL-10 in the 7-d-cultured supernatant of HTLV-BrMMø, in which we found far less IL-10 than in supernatants from 7-d-cultured MT-2 or B95-a (Fig. 2D, middle panels). Nevertheless, the 7-d-cultured supernatant of HTLV-BrMMø containing an undetectable amount of IL-10 significantly inhibited GM-CSF- and IL-4-mediated induction of CD1a expression, although culture supernatants from B95-a, containing more than 30 ng/mL of IL-10, did not inhibit CD1a expression (Fig. 2D, lower panels). As far as we have determined, more than 50 ng/mL of recombinant IL-10 was required to suppress CD1a expression (data not shown). Furthermore, anti-IL-10 neutralizing antibody could not restore CD1a expression (Fig. 2D, lower panels). These observations indicate that IL-10 might be not responsible for the impairment of monocyte differentiation seen in our systems. The inhibitory effect was observed in the high-molecular-weight fraction (>100 kDa) in HTLV-BrMMø-sup, and was not abolished when culture supernatants were heated to 90°C for 30 min or treated with trypsin (Fig. 2C and E), indicating that the active substance is heat- and trypsin-stable.

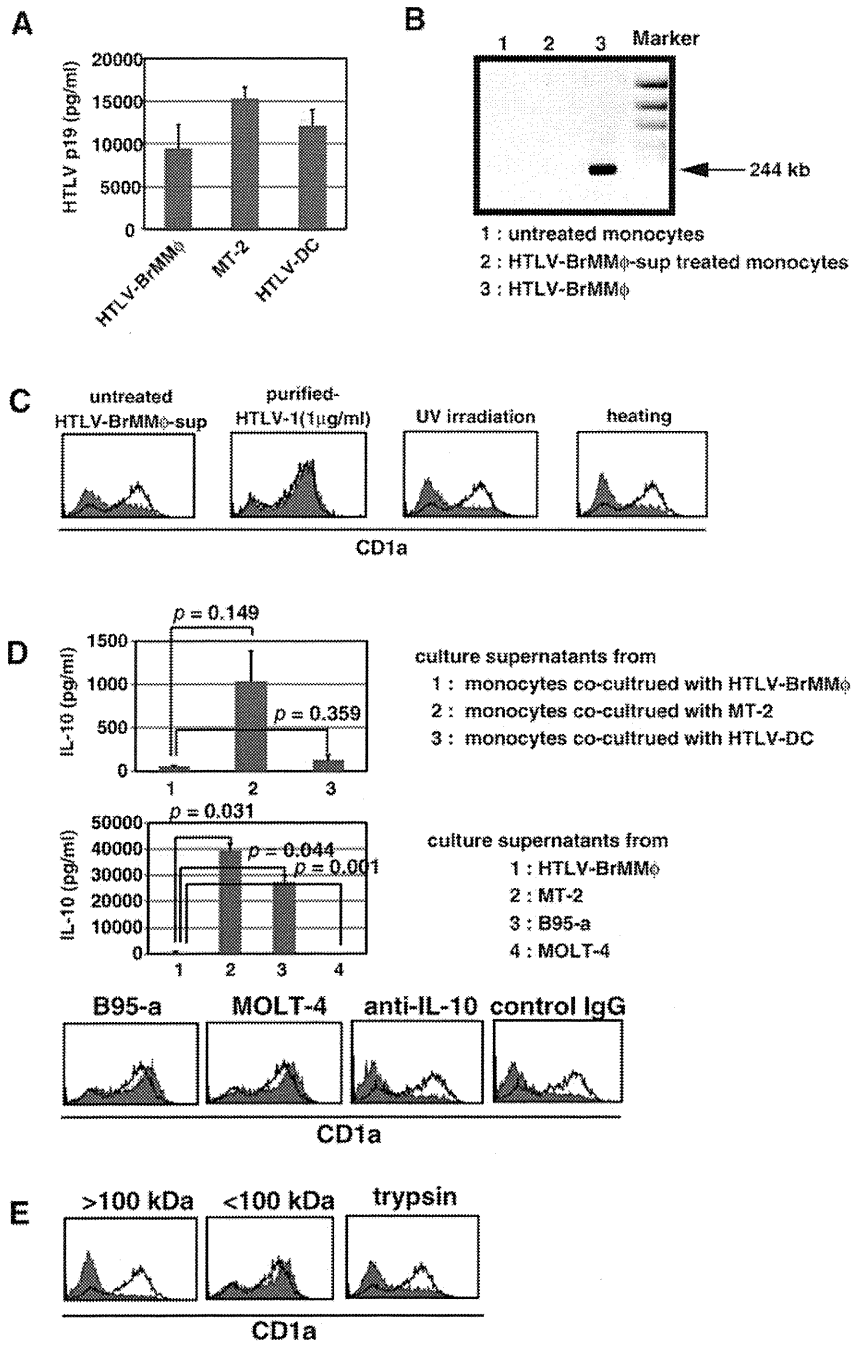

FIG. 2. Characterization of soluble factors responsible for the impairment of monocyte differentiation. (**A**) HTLV-I p19 contents in culture supernatants. $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence of HTLV-BrMMø, MT-2, or HTLV-DC. After 3 d, culture supernatants were harvested and HTLV-I p19 contents were measured by ELISA. (**B**) $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence or absence of HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4. After 2 d, DNA was isolated from monocytes and subjected to PCR analysis. (**C**) $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence of 1 μg/mL purified HTLV-I virions, UV-irradiated, heated HTLV-BrMMø-sup, or untreated HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4. Flow cytometric analysis was performed on day 3. (**D**) IL-10 contents in culture supernatants. $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence of HTLV-BrMMø, MT-2, or HTLV-DC. After 3 d, culture supernatants were harvested and IL-10 contents were measured by ELISA. Thirty thousand cells/mL HTLV-BrMMø, MT-2, B95-a, and MOLT-4 cells were cultured in CTM. After 7 d, culture supernatants were harvested and IL-10 contents were measured by ELISA. $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence or absence of supernatants from either B95-a or MOLT-4, and of 100 mg/mL anti-IL-10-neutralizing antibody or class-matched control IgG. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4. Flow cytometric analysis was performed on day 3. (**E**) HTLV-BrMMø-sup was fractionated by Amicon Ultra-15 or treated with trypsin.

Effect of HTLV-BrMMø-sup on the function of APCs

Primary T-cell responses are required for the interaction between T cells and APCs. Thus we tested the effect of HTLV-BrMMø-sup on the stimulatory capacity of APCs for T-cell proliferation in an MLR. When monocytes or PBMCs were treated with or without HTLV-BrMMø-sup and used as a source of APCs, the stimulatory activity of both cells was significantly reduced (Fig. 3A).

Immature DCs can produce large amounts of cytokines, such as IL-6 and IL-12, when stimulated with LPS or CD40 ligand (CD40L). IL-12 is the most important cytokine in the initiation of Th-1 immunity to virus infection; therefore, monocytes treated with or without HTLV-BrMMø-sup were tested for IL-12p70 production after CD40L stimulation (30 ng/mL; PeproTech, Rochy Hill, NJ). $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence or absence of HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4 for 7 d, and then stimulated with CD40L. After 2 d, we harvested culture supernatants and measured the level of IL-12p70 by ELISA. It was found that untreated monocytes showed increased secretion of IL-12p70 and upregulation of CD83 in response to CD40L stimulation, while CD40L-induced production of IL-12 and upregulation of CD83 was significantly suppressed in HTLV-BrMMø-sup-treated monocytes (Fig. 3B).

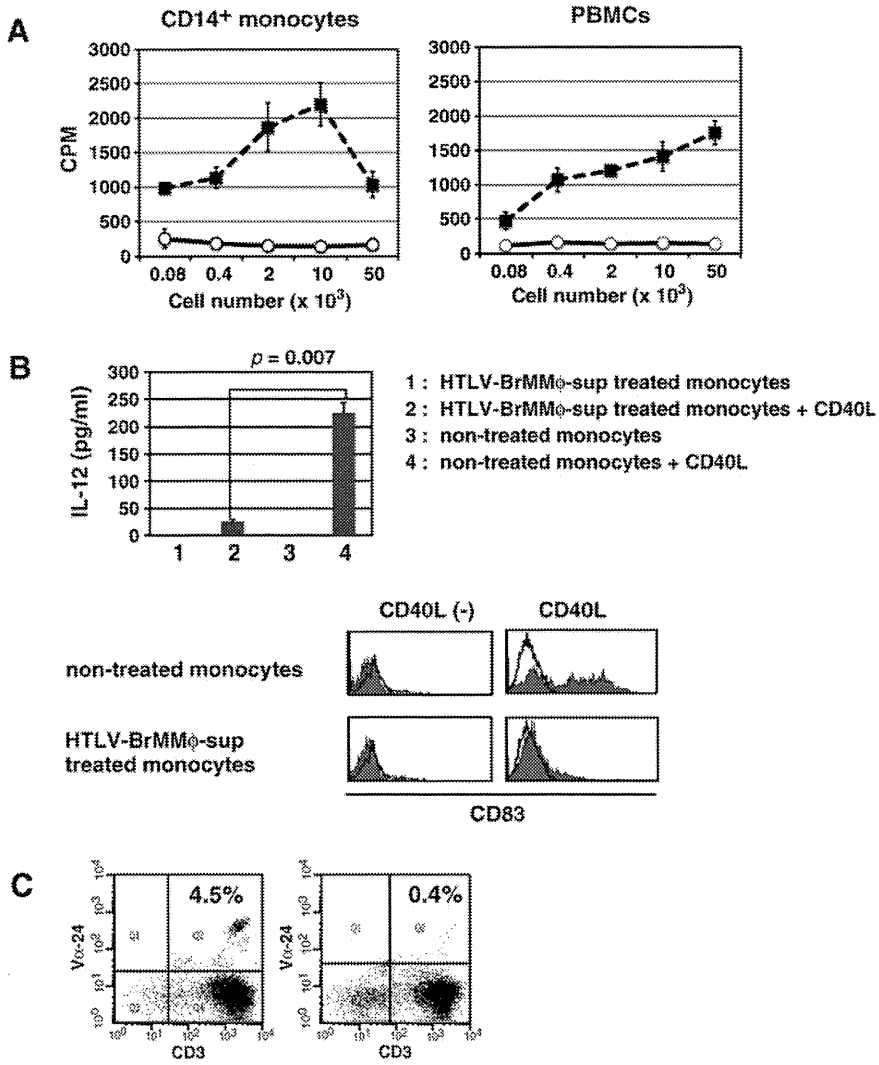

FIG. 3. Effect of HTLV-BrMMø-sup on the function of APCs. (**A**) Stimulatory capacity for T-cell proliferation. Both $CD14^+$ monocytes (left graph) and PBMCs (right graph) were cultured with (open circles) or without (closed squares) HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further incubated in CTM supplemented with GM-CSF and IL-4 for 3 d and were used as APCs. Purified allogeneic T cells were cultured with the indicated number of APCs for 5 d. (**B**) Stimulatory capacity for DC maturation. $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence or absence of HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further incubated in CTM supplemented with GM-CSF and IL-4 for 7 d, and then stimulated with CD40L. After 2 d, the cells and supernatants were used for measurement of IL-12p70 production and CD83 expression. (**C**) Stimulatory capacity for iNKT induction. PBMCs were cultured with 10 ng/mL α-GalCer, either in the presence (right) or absence (left) of HTLV-BrMMø-sup for 7 d. Cell cultures were fed by replacing a half-volume of media every other day with fresh CTM supplemented with IL-2.

It was observed that the expression level of CD1d on monocytes co-cultured with both HTLV-BrMMø and HTLV-BrMMø-sup was slightly upregulated. Invariant natural killer T (iNKT) cells are a subset of lymphocytes that bridge the innate and adaptive immune systems and are potent regulators of diverse immune responses. iNKT cells can become activated directly via engagement of invariant TCR with glycolipid antigens loaded onto CD1d molecules on APCs, or indirectly via activated APCs; therefore, we examined the effect of HTLV-BrMMø-sup on the stimulatory capacity of APCs for the induction of CD1d-restricted iNKT cells by α-GalCer. PBMCs were cultured in CTM with α-GalCer in the presence or absence of HTLV-BrMMø-sup for 7 d and further cultured with IL-2 for additional 7 d. As shown in Fig. 3C, contrary to our expectations, the percentage of $V\alpha24^+$ $CD3^+$ cells was significantly decreased in HTLV-BrMMø-sup-treated PBMCs.

Effect of culture supernatants from MT-2 and HTLV-DCs on monocyte differentiation to immature DCs

Co-culture of monocytes with both uninfected BrMMø and culture supernatants from uninfected BrMMø did not affect monocyte differentiation to DCs (Fig. 1B and D). It was speculated that HTLV-I infection of BrMMø might lead to the production of inhibitory factors; therefore, we further tested the ability of culture supernatants to inhibit monocyte differentiation to DCs in other HTLV-I-infected cells, MT-2, and HTLV-DCs. Predictably, both culture supernatants from MT-2 (Fig. 4A) and HTLV-DCs (Fig. 4C) exhibited the same inhibitory effect as those of HTLV-BrMMø on the expression level of surface molecules. These inhibitory effects were not found in culture supernatants from the HTLV-I-uninfected T-cell line, MOLT-4 (Fig. 4B), or immature DCs (iDCs) (Fig. 4D). ◀F4

Effect of HTLV-BrMMø-sup on differentiated DCs

It was important to evaluate whether HTLV-BrMMø-sup also affected already-differentiated DCs. We prepared iDCs by culturing $CD14^+$ monocytes in the presence of GM-CSF and IL-4 for 7 d, and then cultured with HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4 for 2 d. As shown in Fig. 5, treatment of iDCs with HTLV-BrMMø-sup resulted in little significant alteration of the expression of most surface molecules; however, the expression level of ◀F5

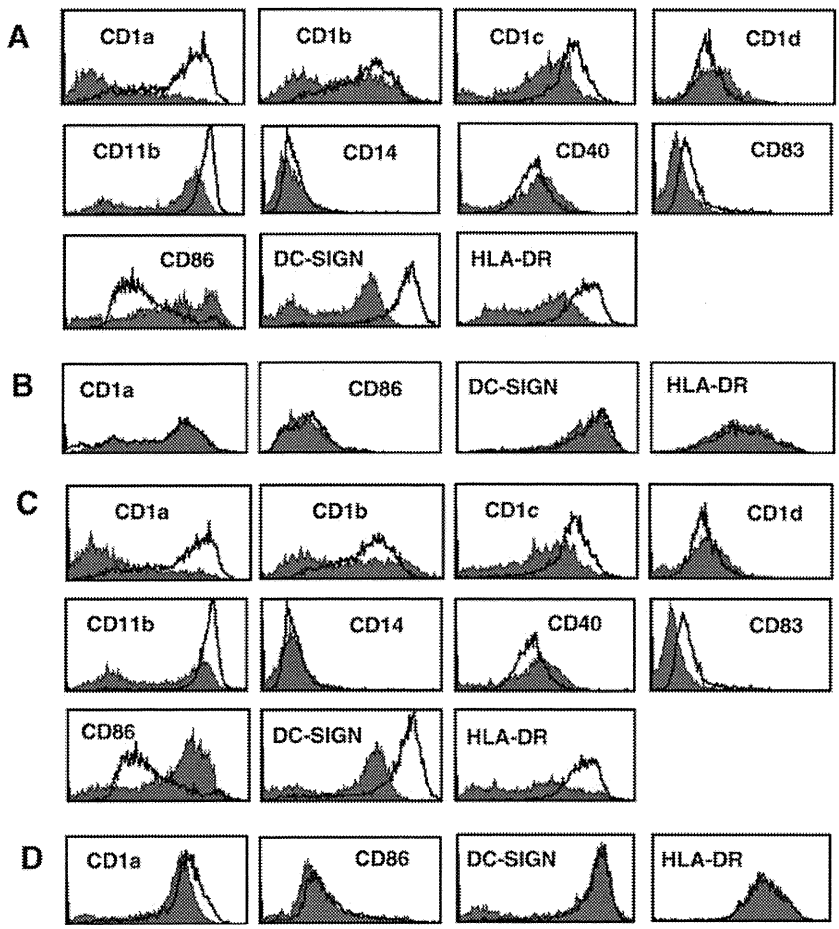

FIG. 4. Effect of culture supernatants from MT-2 and HTLV-DCs on monocyte differentiation to immature DCs. $CD14^+$ monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence or absence of culture supernatants from MT-2 (**A**), MOLT-4 (**B**), HTLV-DCs (**C**), or iDCs (**D**) at a final concentration of 20% for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4. Flow cytometric analysis was performed on day 3.

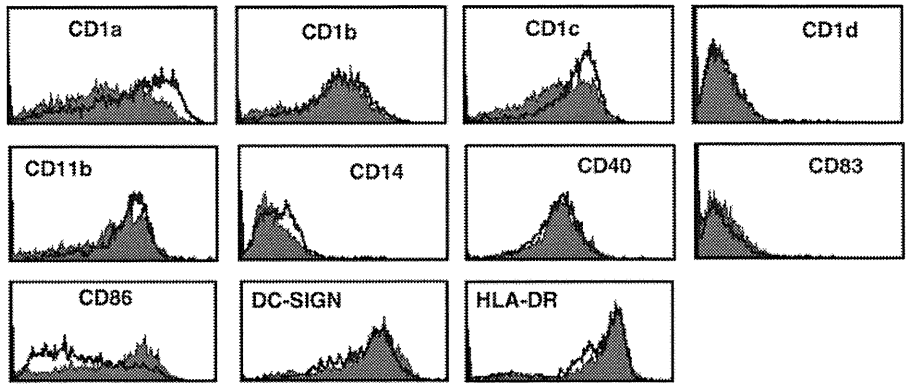

FIG. 5. Effect of HTLV-BrMMø-sup on differentiated DCs. Immature DCs (iDCs) were prepared by culture of $CD14^+$ monocytes in the presence of GM-CSF and IL-4 for 7 d with or without HTLV-BrMMø-sup for 18 h. After washing with PBS, the cells were further cultured in CTM supplemented with GM-CSF and IL-4 for 3 d.

CD86 remained upregulated. These data implied that HTLV-BrMMø-sup affected an early process in monocyte differentiation to DCs.

Discussion

DCs play a critical role in initiating and regulating immune responses by promoting antigen-specific T-cell activation. The ability of DCs to initiate an immune response depends on their transition from antigen-processing to antigen-presenting cells by upregulating MHC class II and T-cell co-stimulatory molecules on the cell surface (24). This transition is an important process in mounting an immune response because immature DCs not only fail to prime T cells effectively, but also serve to promote tolerance induction (34). Makino *et al.* have reported that monocyte-derived immature DCs donated by ATL patients were downregulated in the expression of HLA-DR, CD1a, and CD86 (22). Several other studies have also shown the impairment of DC differentiation in HTLV-I-infected individuals. In our *in vitro* study, we showed that co-culture of monocytes with HTLV-I-infected cells inhibited monocyte differentiation to DCs. This inhibitory effect could be partially recapitulated by incubation with HTLV-BrMMø-sup. Factors leading to DC dysfunction are postulated as follows: first, the direct HTLV-I infection of monocytes; second, the presence of HTLV-I proteins that might modulate DC differentiation; and third, the production of cytokines from HTLV-I-infected cells that might modulate DC differentiation.

First, we tested whether impairment of DC differentiation is required for HTLV-I infection of monocytes. When monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence of UV-irradiated or heated HTLV-BrMMø-sup, the expression levels of CD1a on monocytes remained downregulated. Moreover, we could not detect HTLV-I proviral DNA in any of the samples of monocytes that exhibited impaired differentiation to DCs; therefore, we assumed that the impairment of DC differentiation does not necessarily require HTLV-I infection of monocytes.

The viral protein Tax is known to play a critical role in the genesis of ATL by functioning as a transcriptional activator and an oncoprotein (13). Tax is required for viral replication and is of special importance with respect to HTLV-I-specific immune responses. Jain *et al.* have shown that Tax promoted the production of proinflammatory cytokines and chemokines involved in the DC activation process. Consequently, Tax can induce the expression of CD40, CD80, CD83, and CD86 on DCs, and enhance the T-cell proliferation capability of DCs (17); therefore, Tax appears not to be directly involved in the impairment of DC differentiation. The HTLV-I accessory protein $p12^I$ has been shown to downmodulate the surface expression of HLA-ABC, ICAM-1, and ICAM-2 (6,18). We elevated the expression levels of $p12^I$ in CD1a-expressing cells using $p12^I$ expression vector (kindly provided by Dr. Franchini); however, no substantial differences were found in the expression level of CD1a between $p12^I$ expression vector-transfected cells and control vector-transfected cells (data not shown).

Regarding the impairment of DC differentiation, some studies have proposed mechanisms by which soluble products from various types of cells interfere with DC differentiation. Cytokines produced by tumor cells such as IL-1β, IL-6, IL-10, and vascular endothelial growth factor (VEGF), are known to suppress DC development and function (12,27). Spaggiari *et al.* have reported that mesenchymal stem cells exhibited an inhibitory effect on DC maturation through the production of prostaglandin E_2 (33). Some studies suggested that IL-10 might be one tool to modulate the immune response in HTLV-I-infected patients (9,22). In this study, we observed that culture supernatants from MT-2 and B95-a cells contained a large amount of IL-10, whereas those from HTLV-BrMMø contained little IL-10; however, culture supernatants from B95-a did not inhibit GM-CSF- and IL-4-mediated induction of CD1a expression, whereas those from HTLV-BrMMø showed significant inhibition, and furthermore, anti-IL-10 neutralizing antibody could not restore CD1a expression. These findings suggest that IL-10 is not responsible for the impairment of monocyte differentiation to DCs in our systems. Since culture supernatants from MT-2 and HTLV-DCs exhibited a similar inhibitory effect on monocyte differentiation to DCs, we speculated that HTLV-I infection might cause the production of some inhibitory cytokines in infected cells.

Recently Azakami *et al.* reported a decreased frequency of iNKT cells in PBMCs from HAM/TSP and ATL patients (5). We also observed that when PBMCs were cultured with α-GalCer in the presence of HTLV-BrMMø-sup, the percentage

of iNKT cells was significantly decreased. iNKT cells are characterized by a semi-invariant T-cell receptor (TCR) using a unique TCR Vα24Jα18 chain in humans, and by its recognition of α-GalCer loaded onto CD1d molecules. They exert potent killing activity and rapidly produce cytokines and chemokines that influence many other cells of the innate and adaptive immune systems. Thus, iNKT cells have been shown to play a critical role in immune regulation, ranging from the development of autoimmunity to responses to pathogens and tumors (37); therefore, the low number of iNKT cells in HTLV-I-infected patients might result in the insufficient control of HTLV-I-infected cells, and lead to the development of ATL.

We found that treatment of monocytes with HTLV-BrMMø-sup exhibited potent inhibitory effects on both T-cell and iNKT-cell proliferation. An effective T-cell response reflects the presence of necessary co-stimulatory and proinflammatory cytokines, such as IL-12, whereas suppression of T-cell responses reflects the absence of co-stimulation and the presence of inhibitory cytokines such as IL-10 (10). When monocytes were cultured in CTM supplemented with GM-CSF and IL-4 in the presence of HTLV-BrMMø-sup, the expression level of CD1a, DC-SIGN, and HLA-DR was significantly downregulated. Moreover, HTLV-BrMMø-sup-treated monocytes exhibited decreased production of IL-12 after simulation with CD40L; therefore, the failure of T-cell proliferation in MLR might be due to the deficiency of both co-stimulatory and proinflammatory cytokines. However, since the expression level of CD86 was upregulated and that of CD40 was not altered, we have to examine the stimulatory capacity of monocytes for an antigen-specific T-cell response. In addition, although the expression level of CD1d was also slightly upregulated, induction of iNKT cells was suppressed. It is much more likely that antigen-processing and antigen-presenting functions may be damaged in monocytes during the differentiation process. Further studies are required to elucidate the characteristics of impaired monocytes and the relationship between the inhibitory effects of HTLV-I-infected cells on monocyte differentiation and dysfunction of HTLV-I-specific immune responses.

Treatment of iDCs with HTLV-BrMMø-sup resulted in few significant changes in the expression of surface molecules compared to untreated iDCs, except CD86. CD86 is a co-stimulatory molecule that engages with CD28 on T cells and provides T cells with co-stimulatory signals. We do not know why the expression level of CD86 was upregulated by treatment with HTLV-BrMMø-sup. Although the molecular mechanism underlying CD86 regulation remains unclear, a recent study demonstrated that CD86 is ubiquitinated in DCs via ubiquitin ligase, and that ubiquitination plays a key role in CD86 expression (8); therefore, treatment of monocytes with HTLV-BrMMø-sup might result in inadequate ubiquitination of CD86, which would lead to an increase in CD86 expression.

Hasegawa *et al.* have reported that orally HTLV-I-infected rats exhibited weaker HTLV-I-specific cellular immune responses with an increased viral load (14). When HTLV-I-infected BrMMø are ingested with maternal breast milk and arrive at the newborn intestinal tract, they might transmit HTLV-I virions to intestinal lymphocytes and monocytes. Moreover, HTLV-I-infected BrMMø might inhibit the proliferation and differentiation of immune cells, including local innate immune responses. These early disturbances in innate immunity of the newborn intestine may result in a failure to develop HTLV-I-specific immune responses.

In the present study, we showed that soluble products from HTLV-I-infected BrMMø exhibited potent inhibitory effects on both T-cell and iNKT-cell proliferation. This indicated functional impairment in both adaptive and innate immunity. Although we have not closely analyzed the soluble products present in culture supernatants from HTLV-I-infected BrMMø, identifying the factors responsible for the impairment of monocyte differentiation to DCs may be helpful for both understanding HTLV-I pathogenesis, and developing new therapeutic approaches.

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Author Disclosure Statement

No conflicting financial interests exist.

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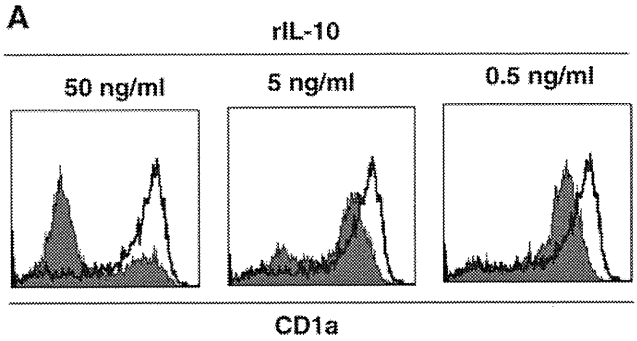
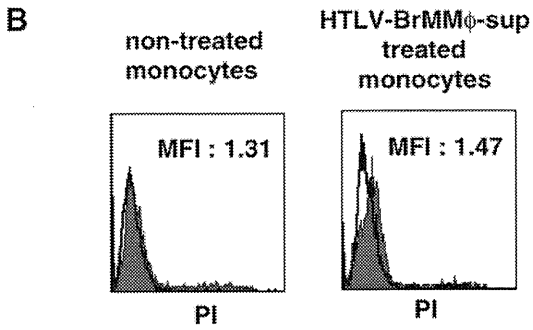
Supplementary Data





AU1 ▶ SUPPLEMENTARY FIG. S1.

SUPPLEMENTARY FIG. S2. ◀AU1

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Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice

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ABSTRACT

Dendritic cells (DCs) play an important role in providing an appropriate fetal/maternal balance between Th1 and Th2 during pregnancy. The Th1/Th2 balance seems to be regulated mainly by two distinct DC subsets, DEC-205$^+$ DCs having the capacity to establish Th1 polarization and 33D1$^+$ DCs to induce Th2 dominance. Pregnancy is established and maintained by maternal hormones, such as progesterone and estrogen, and the balance of DC subtypes was affected mainly by progesterone, which induced a dose-dependent reduction of the DEC-205/33D1 ratio together with/without a stable amount of estrogen. The DEC-205/33D1 ratio decreased gradually with the progress of pregnancy and rapid augmentation of the ratio was seen around delivery *in vivo*. Here, we demonstrate that depletion of 33D1$^+$ DCs during the perinatal period caused substantial fetal loss probably mediated through Th1 up-regulation via transient IL-12 secretion, and pre-administration of progesterone could rescue the fetal loss. Similar miscarriages were also observed when pregnant mice were intraperitoneally (i.p.) injected twice with IL-12 on Gd 9.5 and 10.5. Moreover, prior inoculation of progesterone suppressed the enhanced serum IL-12 production in mice treated with 33D1 antibody, indicating that progesterone might inhibit temporal IL-12 secretion around Gd 10.5 and miscarriage was avoided. These findings suggest the importance of balancing DC subsets during pregnancy and reveal that we can avoid miscarriage by manipulating the activity of the DC subpopulation of pregnant individuals with maternal hormones.

Introduction

The maternal immune response is crucial for a successful pregnancy. Immunity against fetal semi-alloantigens encoded by paternal genes is thought to be tolerated, although it should protect the fetus from infections during pregnancy. Nevertheless, at a suitable time, the fetus is safely delivered from the mother. In this regard, the appropriate balance between Th1 and Th2 has been discussed during pregnancy. Indeed, it is considered that predominant Th2 immunity is essential for maintaining a successful pregnancy (Blois et al. 2004; Piccinni et al. 1998). Thus, under Th2 dominancy, the symptoms of many diseases correlated with autoimmune disorders are altered during pregnancy. For example, systemic lupus erythematosus (SLE), associated with Th2 cytokine production, tends to flare and worsen (Doria et al. 2008), while rheumatoid arthritis (RA), linked to Th1 cytokines, is apt to improve during pregnancy (Doria et al. 2006).

Innate DCs are the most powerful antigen-presenting cells (APCs) and are critical for the induction of primary immune responses. Pattern recognition receptors (PRRs), such as TLRs and lectin receptors on DCs, are important to recognize pathogen-associated molecular patterns (PAMPs) (Steinman 1991). The role of such DCs has also been argued in the feto-maternal immune system (Karsten et al. 2009; Laskarin et al. 2007) and many groups have speculated the actual roles of DCs within the uterus during pregnancy (Bizargity and Bonney 2009; Blois et al. 2007; Collins et al. 2009). Moreover, it has recently been reported that tissue-residing DCs, entrapped in the decidua, might minimize immunogenic T cell activation against fetal/placental antigens in the draining lymph nodes and establish tolerance against the embryo (Collins et al. 2009). Furthermore, it has recently been reported that depletion of uterine DCs altered decidual angiogenesis and caused embryo resorption even in a T cell-deficient pregnant state (Plaks et al. 2008; Pollard 2008). These findings strongly suggest the direct involvement of decidual DCs in the maintenance of pregnancy and reveal the possibility that pregnancy is mediated without specific

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acquired T cell immunity that may cause substantial damage to internal foreign organs, such as transplanted semi-allogeneic grafts (Nagler et al. 2003). Nevertheless, safely delivered newborn babies generally have not suffered any damage during pregnancy even in the semi-allogeneic state, indicating that allogeneic antigen-specific acquired immunity may not be a major factor for delivery. Moreover, the fact that syngeneic mating of mice can produce offspring in the absence of semi-allogeneic antigens in the fetus strongly suggests that allo-specific acquired immunity may be less related to normal delivery than expected; therefore, we examined the actual relationship of innate maternal DCs for the maintenance of pregnancy and the initiation of delivery.

Recently, two major distinct subsets of $CD11c^+$ DCs expressing their own Clec4a4 C-type lectin domain family 4, member a4, 33D1 (Dudziak et al. 2007) and LY75, DEC-205 (CD205) (Bozzacco et al. 2007), have been shown to regulate our immune responses *in vivo* (Figdor et al. 2002). $DEC\text{-}205^+$ DCs have the capacity to establish Th1 polarization, whereas $33D1^+$ DCs, recognizing dendritic cell inhibitory receptor-2 (DCIR2) (Dudziak et al. 2007), create Th2 polarization (Moriya et al. 2010). Based on such findings, we examined the kinetics of the expression of C-type lectin receptors on DCs during the perinatal period and analyzed the influences of these receptor-positive DCs on the maintenance of pregnancy requiring an appropriate Th1/Th2 balance.

In the present study, we studied the kinetics of murine DC subsets, $DEC\text{-}205^+$ and $33D1^+$ DCs, in the spleen during the perinatal period and found sharp augmentation of the DEC-205/33D1 ratio just after delivery, which was mainly caused by the rapid decease of $33D1^+$ DCs and the prompt increase of $DEC\text{-}205^+$ DCs. Surprisingly, apparent fetal loss could be induced reproducibly by the depletion of $33D1^+$ DCs during the perinatal period. We also found that the balance of DC subtypes is profoundly affected by progesterone, which caused an apparent reduction of the DEC-205/33D1 ratio with increasing doses. Thus, we injected progesterone to see whether we could rescue the fetal loss generated through the depletion of $33D1^+$ DCs, and this was confirmed.

These findings demonstrate the importance of balancing the innate DC subsets during pregnancy and suggest that we may rescue miscarriages by manipulating the number and activity of the DC subpopulation in pregnant individuals with maternal hormones.

Materials and methods

Mice

Female BALB/c, male BALB/c, female C57BL/6, and male C57BL/6 mice were purchased from Charles River Laboratories (Kanagawa, Japan), maintained in micro-isolator cages under specific pathogen-free conditions, and fed autoclaved laboratory chow and water with a 12 h light–dark cycle. Virgin females (8–12 weeks) were mated with males. The day when a vaginal plug was detected in the mated females was determined as gestational day (Gd) 0.5. Pregnant mice were sacrificed on Gd 5.5, 10.5, 15.5, 18.5, just after delivery, and 5 days after delivery. Spleen, paraaortic lymph nodes (PALNs), and the uterus were removed from pregnant mice, and cells were harvested from these organs. All animal experiments were performed according to guidelines for the care and use of laboratory animals set by the National Institutes of Health (NIH, MD) and approved by the Review Board of Nippon Medical School (Tokyo, Japan).

Cell preparation and flow cytometry analysis

The decidua of the uterus was removed together with the placenta using ophthalmic scissors under a stereoscopic microscope. After removing the spleen, PALNs, and decidua, the tissues were cut into small pieces and incubated with 1 mg/ml collagenase D (Roche, Basel, Switzerland) at 37 °C for 45 min. The obtained cells were finely mashed, passed through nylon mesh, and washed with RPMI 1640 medium (Sigma–Aldrich, St Louis, MO). To eliminate contaminated red blood cells, the cells were suspended with 0.1× PBS (pH 7.2, 0.15 M NaCl) for osmotic hemolysis and an equal amount of 2× PBS was added immediately after lysis. Cells were centrifuged (1200 rpm, 20 °C, 8 min) and collected. After incubation with Fc blocker (clone 24G2; American Type Culture Collection (ATCC), Manassas, VA) for 15 min to avoid non-specific antibody binding with Fc receptors, the cells were stained for 30 min at 4 °C with 50 μl diluted FITC- or PE-labeled monoclonal antibodies (mAbs): FITC-conjugated anti-DEC-205 (clone: NLDC-145 (rat IgG2a); Cedarlane, Ontario, Canada), PE-conjugated 33D1 (clone: 33D1 (rat IgG2b); BD Biosciences, San Diego, CA), FITC-conjugated anti-CD11c (clone: N418; BD Biosciences), PE-conjugated anti-CD11c (clone: N418; BD Biosciences), FITC-conjugated anti-CD11b (clone: M1/70; eBioscience, San Diego, CA), PE- and FITC-conjugated anti-CD8α (clone: 53-6.7; BioLegend, San Diego, CA), FITC-conjugated anti-CD4 (clone GK1.5; eBioscience), PE-conjugated anti CD4 (clone: RM4.5; BD Biosciences), APC-conjugated anti-CD3 (clone 145-2C11; BioLegend), Biotin-conjugated anti CD69 (clone: H1.2F3; BD Biosciences), APC Cy7-conjugated Streptavidin (BD Biosciences), and FITC-conjugated anti-CD25 (clone 7D4; BD Biosciences). After washing twice with FACS buffer solution, dead cells were determined using propidium iodide (PI; Sigma–Aldrich) or 7-amino-actinomycin D viability dye (Beckman Coulter), and stained cells were analyzed with FACScan using the CellQuest program (BD Biosciences) or analyzed by FACScanto II (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR). DCs were gated according to their FSC/SSC characteristics. PE- and FITC-labeled rat IgG2a isotype-matched control antibodies (clone: RTK2758; BioLegend) and rat IgG2b isotype-matched control antibodies (clone: RTK4530; BioLegend) were used as negative controls to set the gate thresholds of each measurement.

Effect of progesterone and estradiol on bone marrow-derived DCs

Bone marrow cells were obtained from the femur of virgin female BALB/c mice. Cells suspended in RPMI 1640 were centrifuged (1500 rpm, 20 °C, 5 min) and collected. The cells were then re-suspended in 0.1× PBS for osmotic hemolysis and an equal amount of 2× PBS was added immediately to eliminate contaminating red blood cells. Cells were centrifuged (1500 rpm, 20 °C, 5 min) and collected. Obtained bone marrow cells were cultured at a density of 1×10^6 cells in 1 ml RPMI-1640-based culture medium (Takahashi et al. 1996) supplemented with 2 mM L-glutamine (Sigma–Aldrich), 1 mM sodium pyruvate (Invitrogen Life Technologies, Carlsbad, CA), 0.1 mM nonessential amino acid (Invitrogen Life Technologies), a mixture of vitamins (Invitrogen Life Technologies), 1 mM HEPES (Invitrogen Life Technologies), 100 U/ml penicillin (Invitrogen Life Technologies), 100 μg/ml streptomycin (Invitrogen Life Technologies), 50 mM 2-ME (Sigma–Aldrich) and heat-inactivated 10% fetal calf serum (FCS; Hyclone, Logan, UT) (ref), containing 10 ng/ml GM-CSF (Biosource International, Camarillo, CA) and 10 ng/ml IL-4 (Biosource International, Inc.) in 24-well flat-bottom multiwall plates (BD Biosciences) at 37 °C. Progesterone or estradiol (both from Sigma–Aldrich) was added at the following concentrations: 0.1 μg/ml, 1 μg/ml, and 3 μg/ml at the time of starting culture. On day 2, the culture medium was replaced with fresh medium and the floating cells (e.g. lymphocytes) were removed. On day 5, floating and loosely adherent cells were collected, washed, and analyzed by FACScan.

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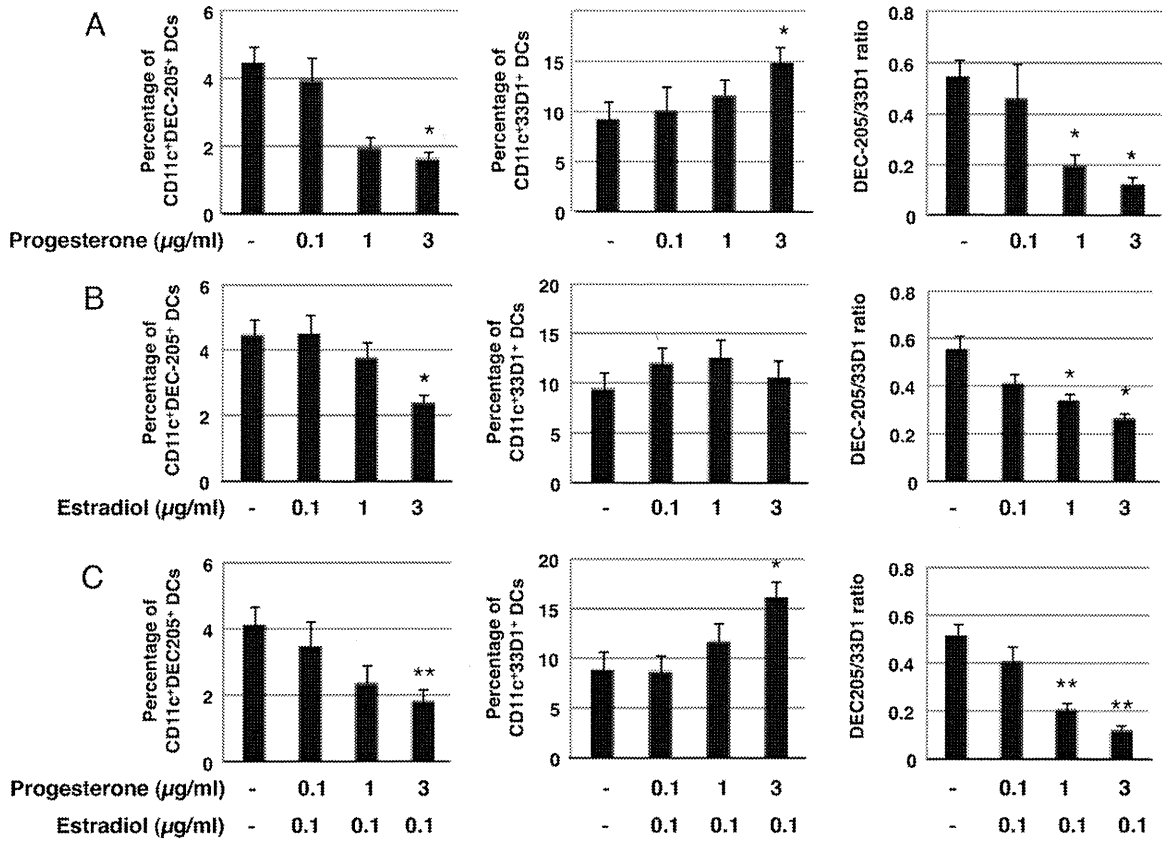

Fig. 1. Kinetics of CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs derived from bone marrow cells co-cultured with progesterone and/or estradiol. The data were obtained from cytometric analysis. (A) Kinetics of CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs co-cultured with progesterone (μg/ml), (B) with estradiol (μg/ml), and (C) with progesterone (μg/ml) and estradiol (0.1 μg/ml). Data are shown as the mean ± SEM of $n = 7$–9 mice per group. $^*p < 0.05$ and $^{**}p < 0.01$.

Analysis for the expression of progesterone receptors (PRs) and estrogen receptor alpha (ERα) in DCs

The expression of progesterone receptors (PRs) and estrogen receptor alpha (ERα) in DCs was analyzed by intracellular staining of DCs. First, cells were stained with FITC- or PE-conjugated anti-CD11c to mark DCs and the marked cells were fixed with Fixation/Permeabilization solution (BD Biosciences) at 4 °C for 20 min. Permeabilized DCs were further stained with PE-conjugated 33D1 antibody or anti-progesterone receptor (PR)-specific antibody (clone: PR-AT 4.14; Abcam, Cambridge, UK) or anti-estrogen receptor alpha (ERα)-specific antibody (clone: 33; Abcam) at 4 °C for 60 min. These antibodies were labeled with Alexa Fluor' dyes (Zenon mouse IgG labeling kits) (Invitrogen, Eugene, OR). After being washed twice, cells were re-suspended in FACS buffer solution and analyzed by FACScanto II (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR).

Induction of fetal loss by 33D1 antibody or by recombinant murine IL-12p70 injection

To investigate the effect of the imbalance of DC subsets on fetal loss, CD11c$^+$33D1$^+$ DCs were depleted from pregnant mice by three intraperitoneal (i.p.) injections on Gd 5.5, 6.5, and 7.5 with 0.5 mg 33D1 antibody (mAb against DCIR2) purified by ion-exchange chromatography using diethylaminoethyl (DEAD) cellulose (DE52; Whatman, Maidstone, UK). As controls, pregnant mice were i.p. injected with 0.5 mg control isotype-matched rat IgG2b (Jackson Immuno Research Laboratories, West Grove, PA). The depletion of CD11c$^+$33D1$^+$ DCs in the spleen was confirmed using FACScan. For IL-12 administration, pregnant mice were i.p. injected with murine recombinant IL-12p70 (0.2 μg/mouse) (R & D Systems, Minneapolis, MN) twice on Gd 9.5 and 10.5. Miscarriage on Gd 16.5 was identified macroscopically as a dark, small fetus. The percentage of fetal loss was calculated as follows: (resorbed implantation numbers/sum of viable and resorbed implantation numbers) × 100%. To examine the effect of progesterone on protection against miscarriage, mice were treated with i.p. injection of 3 μg progesterone (Sigma–Aldrich) on Gd 2.5, 3.5, and 4.5, followed by i.p. injection of 0.5 mg 33D1 antibody on Gd 5.5, 6.5, and 7.5.

Effect of IL-13 injection on rescue of fetal loss induced by IL-12p70 injection

To clarify the role of IL-13, recombinant IL-13 (0.02 μg/mouse) (R & D Systems) was i.p. injected simultaneously with IL-12p70 injection and the percentage of fetal loss was calculated.

Cytokine detection

Cytokine production in the sera from the heart of each pregnant mouse was measured at various time points. The IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13 levels of each sample were measured using a FlowCytomix Simplex Kit (eBioscience). Also, the levels of IL-12p40 as well as IL-12p70 were measured using specific ELISA kits (R & D Systems).

Statistical analysis

The results were analyzed using Student's *t*-test and the results are presented as the mean ± SEM. Differences at $p < 0.05$ were considered significant.

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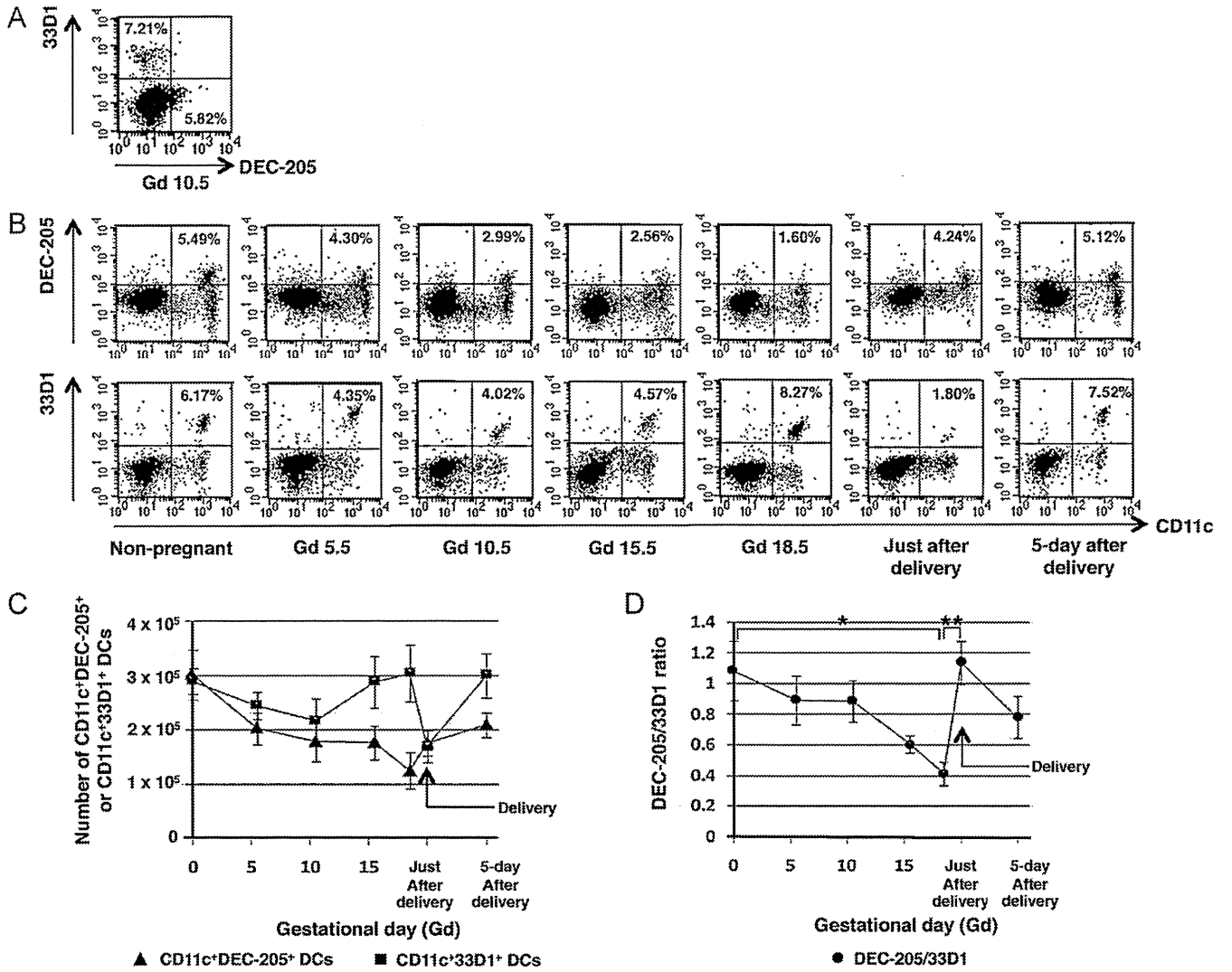

Fig. 2. Flow cytometric analysis of CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs during perinatal period in the spleen. (A) Distribution of DEC-205$^+$ (FITC-labeled) and 33D1$^+$ (PE-labeled) DCs in spleen on Gd 10.5. Double-positive DCs, DEC-205$^+$33D1$^+$ DCs, were not detected in spleen. (B) Typical flow cytometric analysis of CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs during perinatal period. Divided by the cross in each figure, the upper right region was double-stained for FITC- and PE-conjugated mAb. DCs were gated according to their FSC/SSC characteristics. (C) Number of CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs during perinatal period. Delivery occurred on Gd 19.5 or 20.5 for all mice. (D) Alteration of DEC-205/33D1 ratio during perinatal period. Values in (D) were calculated from the values in (C). Data are shown as the mean ± SEM of $n = 6$–10 mice per group. $^*p < 0.05$ and $^{**}p < 0.01$.

Results

Effects of progesterone and estrogen (estradiol) on the development of DC subsets derived from bone marrow cells

Various surface markers on DCs enabled us to classify their subpopulations. Among them, two major non-overlapping distinct subsets of DCs expressing their own C-type lectin receptors, 33D1 and DEC-205, have recently been found to regulate internal immune responses (Figdor et al. 2002). DEC-205$^+$ DCs establish a Th1 predominant state while 33D1$^+$ DCs, which recognize DCIR2, induce Th2 polarization (Dudziak et al. 2007). Also, the role of such DCs in the regulation of proper Th1/Th2 balance during pregnancy has been addressed (Laskarin et al. 2007). Pregnancy is established and maintained by maternal hormones, such as progesterone and estrogen, particularly progesterone. Indeed, the serum progesterone concentration increases up to 0.3 μM during pregnancy (Virgo and Bellward 1974).

To clarify the effect of progesterone and estradiol on the development of DCs, we analyzed the expressions of DEC-205 and 33D1 on CD11c$^+$ DCs generated from murine bone marrow cells co-cultured with progesterone and/or estradiol. When bone marrow cells were stimulated with murine GM-CSF and IL-4 in the presence of progesterone, the percentage of CD11c$^+$DEC-205$^+$ DCs was markedly reduced, while that of CD11c$^+$33D1$^+$ DCs was increased, and thus the DEC-205/33D1 ratio was apparently decreased (about 0.54–0.13, $p < 0.05$) in a dose-dependent manner (Fig. 1A); however, when bone marrow cells were co-cultured in the presence of estradiol, the percentage of CD11c$^+$33D1$^+$ DCs was almost unchanged, although the percentage of CD11c$^+$DEC-205$^+$ DCs was partially reduced (Fig. 1B), and the DEC-205/33D1 ratio was decreased to some extent (about 0.54–0.26, $p < 0.05$).

In general, the serum concentration of both progesterone and estradiol increases during pregnancy, with the former much higher than the latter (Virgo and Bellward 1974). Therefore, we examined the synergistic effect of progesterone and less estradiol on DC development. When bone marrow cells were co-cultured with progesterone together with 0.1 μM estradiol, the DEC-205/33D1 ratio was more stably and significantly decreased (0.51–0.11, $p < 0.01$) with an apparent reduction of CD11c$^+$DEC-205$^+$ DCs and increase

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of CD11c$^+$33D1$^+$ DCs in a dose-dependent manner (Fig. 1C). To determine the mechanism of how DCs are differently affected by the addition of progesterone and/or estrogen, we examined the intracellular expression of progesterone receptor (PR) and estrogen receptor alpha (ERα) in bone marrow-derived DCs and found that PR and ERα were weakly but almost equally expressed in both CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs (data not shown). The results indicate that the difference in DC growth by progesterone and/or estrogen might not have been caused by the amount of expression in their receptors.

Collectively, progesterone has strong potential to reduce the DEC-205/33D1 ratio in the presence of a small amount of estrogen, such as estradiol, to firmly suppress Th1 dominancy through DEC-205$^+$ DC reduction and 33D1$^+$ DC increase, particularly at more than 1 μg/ml.

Kinetics of DC subsets during pregnancy in the spleen

Among maternal hormones, the serum progesterone concentration increases during pregnancy in the presence of a small amount of estrogen. Thus, we examined the alterations in the number of murine splenic CD11c$^+$ DCs expressing either DEC-205 or 33D1 during pregnancy.

As has been previously demonstrated (Bozzacco et al. 2007; Moriya et al. 2010), CD11c$^+$ murine splenic DEC-205$^+$ or 33D1$^+$ DCs are non-overlapping distinct subsets (Fig. 2A). Based on this notion, we performed flow cytometric analysis to identify CD11c$^+$DEC-205$^+$ (Fig. 2B, upper panels) or CD11c$^+$33D1$^+$ DCs (Fig. 2B, lower panels) in the spleen on successive gestational days (Gds) 5.5, 10.5, 15.5, 18.5, and just after delivery, as well as 5 days after delivery.

As indicated in the figures, the number of CD11c$^+$DEC-205$^+$ DCs decreased during pregnancy and ended with a sudden increase that was determined just after delivery. In contrast, the number of CD11c$^+$33D1$^+$ DCs gradually decreased in the early phase of pregnancy until around Gd 10.5, increased in the late phase of pregnancy, and sharply declined around the delivery date. The sharp reduction in the percentage of CD11c$^+$33D1$^+$ DCs recovered rather quickly after delivery.

To clarify the alteration of the Th1/Th2 balance during pregnancy, the kinetics of the number of either CD11c$^+$DEC-205$^+$ DCs (closed triangle) or CD11c$^+$33D1$^+$ DCs (closed square) was plotted on the basis of flow cytometric analysis (Fig. 2C). The DEC-205/33D1 ratio was calculated on the basis of the above data. As shown in Fig. 2D, although the DEC-205/33D1 ratio (closed circle) rapidly decreased toward the end of pregnancy, a sharp augmentation of the ratio was seen just after delivery, which might have been initiated by the marked increase of CD11c$^+$DEC-205$^+$ DCs together with the rapid decrease of CD11c$^+$33D1$^+$ DCs.

Kinetics of DC subsets during pregnancy in the zone associated with the uterus

The kinetics of DC subpopulations in the PALNs and decidua associated with uterus was also examined. In contrast to the spleen, the number of CD11c$^+$DEC-205$^+$ DCs in PALNs increased before delivery and thus the DEC-205/33D1 ratio was significantly augmented before delivery. Although a number of DC-lineage CD11c$^+$ cells were observed in the decidua, only a few CD11c$^+$DEC-205$^+$ DCs were detected and CD11c$^+$33D1$^+$ DCs were hardly seen (data not shown). We performed similar experiments several times; however, it was difficult to evaluate the kinetics of DC subsets in the decidua quantitatively. Nevertheless, the number of CD11c$^+$DEC-205$^+$ DCs in the PALNs tended to decrease during pregnancy. Such reduction of CD11c$^+$DEC-205$^+$ DCs in PALNs might be beneficial for maintaining pregnancy. Further analysis of DC subsets in the zone associated with the uterus will be needed to clarify the actual roles of DCs in pregnancy.

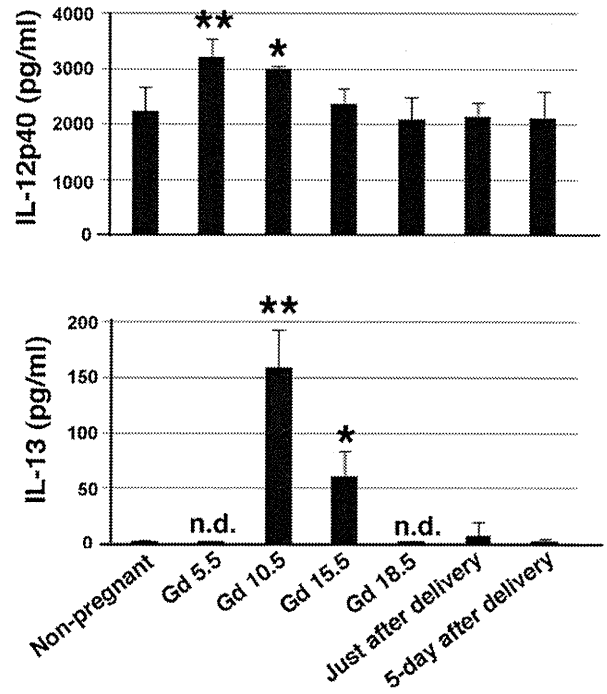

Fig. 3. IL-12p40 (pg/ml) and IL-13 (pg/ml) production in the sera during perinatal period. Data are shown as the mean ± SEM of $n = 6$–10 mice per measurement.

Kinetics of cytokine production in the sera of pregnant mice

Kinetics of the secretion of various cytokines in the sera during pregnancy was determined by FlowCytomix or ELISA. We measured serum IL-2 and IL-12 as Th1 cytokines and IL-4, IL-5, IL-6, IL-10, and IL-13 as Th2 cytokines. Because we hardly detect IL-12p70, a bioactive form of IL-12, in the standard ELISA kit (R & D Systems) in the obtained murine sera, we measured IL-12p40, a sub component of IL-12, instead. As far as we have examined, temporal elevation of IL-12p40 in the early phase of pregnancy followed by IL-13 elevation could be observed in the sera (Fig. 3). IL-2, IL-4, IL-5, IL-6, and IL-10 were not seen in the sera of pregnant mice (data not shown). The results indicate that cytokines produced mainly by innate cells such as DCs (names as "innate cytokines") were dominantly secreted in the syngeneic mating system. It should be noted that a similar secretion pattern of "innate cytokines" was also observed in the allogeneic mating system (BALB/c (female) × C57BL/6 (male)), in which a higher amount of IL-13 was detected during pregnancy (Y.N, M.S., and H.T., unpublished observation).

Depletion of 33D1$^+$ DCs in pregnant mice

The above findings strongly suggest that the sharp augmentation of the DEC-205/33D1 ratio is associated with the initial signaling to halt a pregnancy. This finding prompted us to examine the effect of CD11c$^+$33D1$^+$ DC depletion on fetal loss in pregnant mice. Based on our previous findings (Moriya et al. 2010), pregnant mice were i.p. administrated with 0.5 mg purified 33D1 antibody for 3 consecutive days (Gd 5.5, 6.5, and 7.5). Although CD11c$^+$33D1$^+$ splenic DCs were completely depleted *in vivo* at least two weeks after injection in normal non-pregnant mice (Moriya et al. 2010), such DCs could be observed in pregnant mice less than ten days after administration (Gd 15.5, Fig. 4A), indicating that CD11c$^+$33D1$^+$ DCs seem to recover earlier in pregnant mice. The depletion of

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