Virology **274,** 140–148 (2000)
doi:10.1006/viro.2000.0445, available online at http://www.idealibrary.com on IDEAL®

metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Production of Functionally Deficient Dendritic Cells from HTLV-I-Infected Monocytes: Implications for the Dendritic Cell Defect in Adult T Cell Leukemia

Masahiko Makino,*,†[1] Shin-ichi Wakamatsu,* Satoshi Shimokubo,* Naomichi Arima,‡ and Masanori Baba*

*Division of Human Retroviruses, Center for Chronic Viral Diseases and ‡First Department of Medicine, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan; and †Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan

Received January 10, 2000; returned to author for revision April 19, 2000; accepted May 12, 2000

Adult T cell leukemia (ATL) is induced by an infection with human T lymphotropic virus type I (HTLV-I) and is accompanied by immunodeficiency. Monocyte-derived immature dendritic cells (DCs) donated by 11 ATL patients were suppressed in the ability to take up fluorescein isothiocyanate (FITC)–dextran and were down-regulated in the expression of CD1a and CD86 antigens (Ags). Monocytes from the patients showed impaired expression of CD14 and HLA-DR Ags. These results suggest intrinsic abnormalities of monocytes and a defect of DC maturation in ATL patients. Therefore, we examined the influence of HTLV-I infection of monocytes on their differentiation to DCs. Monocytes obtained from healthy donors were susceptible to HTLV-I infection *in vitro.* HTLV-I-infected monocytes were down-regulated in the expression of CD14 Ags, and immature DCs obtained from them expressed CD1a poorly and were impaired in the ability to take up FITC–dextran. Mature DCs differentiated from these cells could not stimulate autologous $CD4^+$ T cell or $CD8^+$ T cell proliferation, even after being secondarily pulsed with HTLV-I at an immature DC stage. These results suggest that HTLV-I-infected monocytes cannot properly differentiate to DCs and that this might be one of the important mechanisms producing dysfunctional DCs in ATL patients. © 2000 Academic Press

INTRODUCTION

Adult T cell leukemia (ATL) develops 40–60 years after infection with human T lymphotropic virus type I (HTLV-I) in the newborn period (Takatsuki *et al.,* 1977; Uchiyama *et al.,* 1977). During the asymptomatic period of more than 40 years, B cells produce anti-HTLV-I antibodies (Abs), but cytotoxic T lymphocytes (CTL) are considered to control mainly the outgrowth of HTLV-I-infected cells and the expansion of ATL cells. Once ATL develops, both $CD4^+$ and $CD8^+$ T cells show a defect in the proliferative response to various stimuli and it is hard to induce CTL that is specific to HTLV-I Ags *in vitro,* although there are reports of successfully generating CTL from a few ATL patients (Kannagi *et al.,* 1983, 1991). Therefore, the uncontrollable expansion of leukemic T cells and the resulting ATL manifestation seem highly associated with the functional deficiency of lymphocytes including HTLV-I-specific CTL. Among the important mechanisms leading to development of immunodeficiency, the production of functionally impaired antigen-presenting cells (APCs) such as dendritic cells (DCs) or lack of production of proper APCs seems to play an important role. DCs are the most potent APC in that they can stimulate naive and memory $CD4^+$ and $CD8^+$ T cells (Inaba *et al.,* 1987, 1990). The DCs are functionally divided into two major groups. One group includes immature DCs that localize in the blood and peripheral organs and show a distinct ability to internalize various antigens (Ags) through macropinocytosis and the mannose receptor pathway and to process them (Reis e Sousa *et al.,* 1993; Sallusto *et al.,* 1995). In contrast to the immature DCs, mature DCs ensure a strong ability to prime and stimulate T cells specific to an Ag and to allogenic major histocompatibility complex (MHC) molecules. The maturation of DCs is induced by the exposure of immature DCs to inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) and by cell-to-cell contact with activated T cells through CD40 ligand molecules expressed on them (O'Doherty *et al.,* 1993; Roake *et al.,* 1995). However, in the presence of some cytokines such as IL-10, DC maturation is suppressed (Enk *et al.,* 1993; Steinbrink *et al.,* 1997).

In addition to ATL, HTLV-I induces an inflammatory neurogenic disease, HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), in which $CD4^+$ T cells as well as $CD8^+$ T cells are highly activated in an APC-dependent fashion (Osame *et al.,* 1986; Hollsberg and Hafler, 1993). In a previous report, we showed that monocyte-derived DCs were susceptible to HTLV-I infection *in vitro* and *in vivo* in HAM/TSP patients (Makino *et al.,* 1999). The virus-infected DCs stimulate autologous unprimed $CD4^+$ and $CD8^+$ T cells that may be associ-

[1] To whom reprint requests should be addressed. Fax: 81-42-391-8212. E-mail: makino-m@cb3.so-net.ne.jp.

0042-6822/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

ated with the development of HAM/TSP (Makino *et al.,* 1999). In contrast to this inflammatory disease, DCs obtained from tumor-bearing animals are reported to be functionally defective (Chaux *et al.,* 1997; Ishida *et al.,* 1998). In this study, we examined DCs obtained from ATL patients and found that functions of ATL patients' DCs had deteriorated. Furthermore, it has been reported that monocytes are susceptible to HTLV-I infection *in vitro* and *in vivo* in ATL patients (Koyanagi *et al.,* 1993; Wakamatsu *et al.,* 1999). Therefore, we assessed DCs differentiated from peripheral monocytes that were infected with HTLV-I *in vitro* to clarify the immunological mechanisms leading to production of immunocompromised DCs.

RESULTS

Characteristics of DCs obtained from ATL patients

Monocyte-derived DCs were successfully produced from 10 healthy individuals and from 8 patients among the 11 ATL patients examined, although the production rate of DCs from monocytes was reduced in the patients compared to that of normal controls [the DC production rate from $CD2^-$ peripheral blood mononuclear cells (PBMCs): $2.6 \pm 1.1\%$ (ATL, $n = 11$) vs 9.8 ± 2.7 (control, $n = 10$), $P < 0.01$]. Among the healthy controls, there was little variation in all the parameters examined; therefore, we presented only a representative normal donor. The APC function of mature DCs was assessed by using allogeneic mixed lymphocyte reactions (MLRs) (Fig. 1a). We tested two different doses of mature DCs as a stimulator and obtained a similar result. Among the 3 patients examined, one patient's DCs did not stimulate allogeneic unseparated T cells, $CD4^+$ T cells, or $CD8^+$ T cells as strongly as DCs from a normal donor. However, the second patient's DCs showed a reduced ability to stimulate solely $CD4^+$ T cells and the third patient produced DCs that stimulated the T cells to almost the same extent as with a normal donor's DCs. The phenotypic features of the mature DCs from ATL patients were examined (Fig. 1b). All of the DCs expressed CD83 Ags, a marker of mature DCs; however, the expression level of human histocompatibility leukocyte Ag (HLA)-ABC molecules was reduced compared to DCs from a normal donor. However, no substantial differences were found in the expression of HLA-DR, CD40, or CD86 Ags between normal controls and ATL patients (Fig. 1b). In contrast to mature DCs, there were statistically significant differences between normal controls and the patients (Fig. 2). While immature DCs from ATL patients expressed no HTLV-I*gag* Ags in contrast to DCs from HAM/TSP patients (Makino *et al.,* 1999) and expressed HLA-ABC Ags similarly to the DCs from the normal donor, the expression level of CD1a and CD86 Ags was consistently reduced and the level of fluorescein isothiocyanate (FITC)–dextran uptake was down-regulated in the patients' DCs

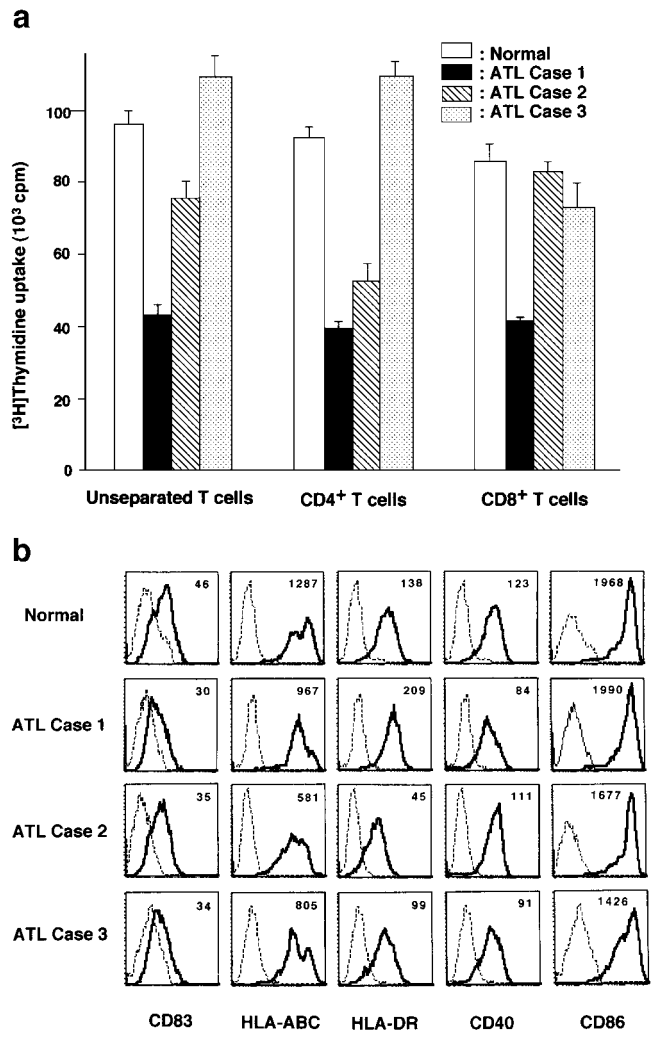

FIG. 1. (a) Allogeneic mixed DC-T cell reactions. Mature DCs were obtained from an uninfected representative normal donor and three ATL patients and were used as stimulator (5×10^3/well). Unseparated $CD4^+$ or $CD8^+$ T cells (1×10^5/well) from a normal donor were used as responders. A 5-day proliferation assay was done in triplicate, and the mean $\pm$ SD is shown. (b) Expression of various molecules on monocyte-derived mature DCs obtained from a healthy donor and ATL patients. (- - -) Control mAb; (—) indicated mAb. The number in each figure represents the mean fluorescence intensity. There were no statistical differences between ATL patients and normal controls in all parameters examined.

(Fig. 2). We did not test viral infection to DCs from patients by polymerase chain reaction (PCR) because both PBMCs and T cells from the ATL patients were actually found to be infected with HTLV-I by PCR analysis (not shown) and possible contamination of a small number of such infected cells made it extremely difficult to determine viral infection to DCs.

Phenotypic features of precursor monocytes from ATL patients

The defects found in the immature DCs from ATL patients might be due to abnormalities of precursor

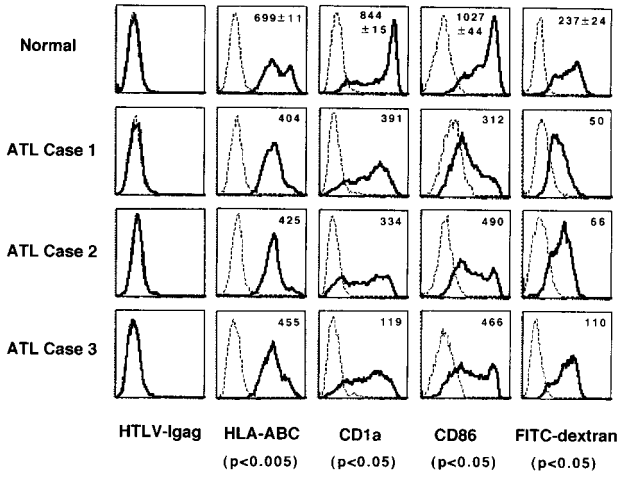

FIG. 2. Expression of HTLV-I Ags and various molecules and the level of FITC–dextran taken up by immature DCs. The immature DCs from healthy donors and ATL patients were differentiated by using rGM-CSF and rIL-4 for 4 days. A representative normal donor is shown. (- - -) Control mAb; (—) indicated mAb or FITC–dextran. The number in each figure represents the mean fluorescence intensity (mean ± SD was calculated from three healthy donors). Statistically significant differences between normal individuals and ATL patients were calculated by using Student's *t* test.

monocytes. We cultured PBMCs for 60 min after depletion of $CD2^+$ T cells and obtained freshly adhering cells. Among the freshly adhered cells, 9.3 ± 1.6% cells from a normal donor expressed CD14 molecules; however, the cells from ATL patients did not express the CD14 Ags as strongly as those from a normal donor ($P < 0.05$ in the mean fluorescence intensity) (Fig. 3). Almost all CD14 molecules on the patients' cells seemed to be masked, because when the cells were cultured for 5 days, they expressed CD14 molecules. However, the expression levels of HLA-DR molecules on the cultured cells from ATL patients were lower than those from the normal donor (Fig. 3, $P < 0.01$ in the mean fluorescence intensity).

Influence of HTLV-I infection on monocytes from normal donors

To clarify the mechanisms leading to the abnormalities found in ATL patients, we examined the effect of HTLV-I infection on monocytes. Monocytes from a normal donor were pulsed with twenty 50% cell culture infective doses ($CCID_{50}$) of cell-free HTLV-I and cultured for 5 days. Using primers corresponding to the HTLV-I*gag* or *px* regions, proviral DNA was detectable as a faint signal by PCR (not shown) and as a strong band by nested PCR from day 1 to day 5 after infection (Fig. 4a). The proviral DNA was still detectable after treatment with RNase (not shown). The viral infection of monocytes from a normal donor induced phenotypic changes in them (Fig. 4b). Monocytes infected by using cell-free HTLV-I or by cell-to-cell contact with MT-2 cells expressed HTLV-I*gag* Ags and had a reduced level of CD14 molecules on their surface at day 5 of culture compared to monocytes pulsed with controls (inactivated virus or Jurkat cells) (Fig. 4b). HLA-DR Ag expression was not influenced by the virus infection (Fig. 4b).

Abnormalities of DCs differentiated from HTLV-I-infected monocytes

To examine the influence of viral infection on normal donor-derived monocytes in terms of DC differentiation, immature and mature DCs were differentiated from HTLV-I-infected monocytes. Genomic DNA from both immature and mature DCs from virus-infected monocytes contained an HTLV-I proviral DNA detectable as a faint band by primary PCR (not shown) and as a strong band by nested PCR (Fig. 5a). Phenotypically the expression of CD1a molecules on the immature DCs from monocytes infected by using cell-free virus or MT-2 cells was down-regulated compared to those from control Ag-pulsed monocytes and the expression of CD86 Ag was reduced when immature DCs were differentiated from monocytes infected by cell-to-cell contact with MT-2 cells (Fig. 5b). The level to take up FITC–dextran was reduced in immature DCs from monocytes infected by using both viral

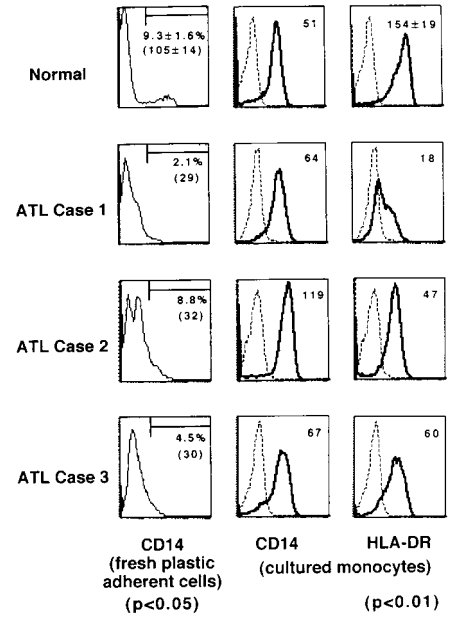

FIG. 3. Expression of CD14 and HLA-DR Ags on fresh plastic adherent and cultured monocytes. Monocytes were obtained from three healthy donors and ATL patients. Plastic adherent monocytes were obtained by culturing CD2 depleted PBMCs for 60 min and were removed with a cell scraper. The adherent monocytes were cultured for 5 days in the presence of 20% fetal calf serum. (- - -) Control mAb; (—) indicated mAb. The percentage represents the percentage of positive cells and the numbers in parentheses and the numbers in each panel for cultured monocytes represent mean fluorescence intensity. Mean ± SD was calculated from three healthy donors, and statistically significant differences between normal individuals and ATL patients in the mean fluorescence intensity were calculated by using Students' *t* test.

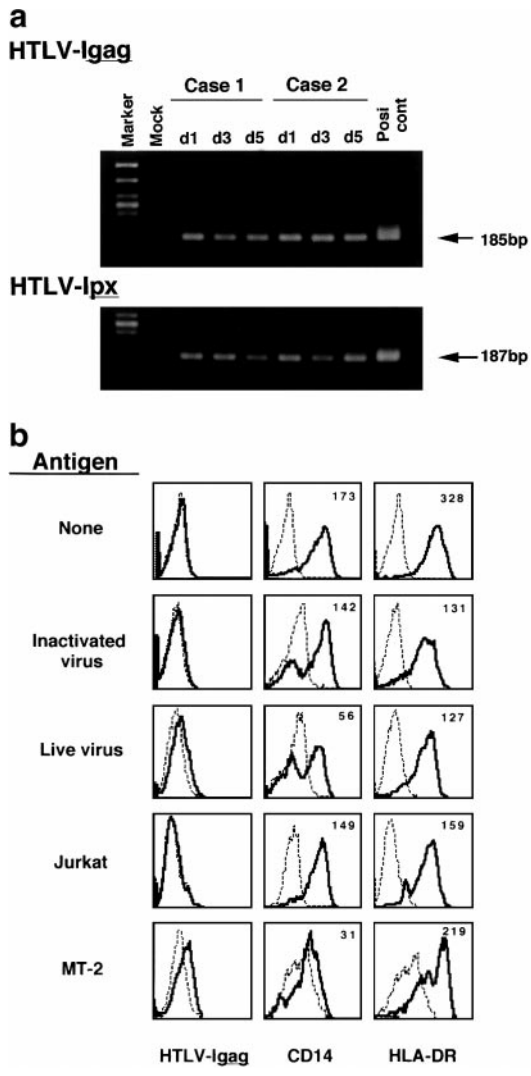

FIG. 4. (a) Detection of HTLV-I proviral DNA by nested PCR. Plastic adherent monocytes from healthy donors were pulsed with 20 $CCID_{50}$ of cell-free HTLV-I or an equivalent dose of heat-inactivated virus and cultured for 5 days. Genomic DNA was extracted from mock- or HTLV-I-infected monocytes on days 1, 3, and 5 after culture and amplified by PCR. (b) Expression of various molecules on cultured monocytes. Monocytes were obtained from a healthy donor and the same monocyte population was pulsed with indicated Ags and cultured for 5 days. Twenty $CCID_{50}$ of HTLV-I and/or the equivalent dose of heat-inactivated virus and 2×10^5 cells/well of Jurkat or MT-2 cells were used as antigens. A representative of three independent experiment is shown.

sources (Fig. 5b). However, the phenotype of mature DCs from virus-infected monocytes was not affected in the expression of molecules including CD40, CD86, and HLA-DR Ags (not shown). In contrast to these phenotypic features, the autologous T cell-stimulating function of mature DCs obtained from the virus-infected monocytes was affected. Mature DCs infected with HTLV-I at a stage of immature DCs, but not at day 0 of the monocyte stage, strongly stimulated autologous $CD4^+$ T cells and $CD8^+$ T cells as described previously (Makino *et al.,* 1999); however, mature DCs differentiated from HTLV-I-infected monocytes did not stimulate autologous T cells despite being infected with the virus (not shown). Various amounts of virus (up to 100 $CCID_{50}$ cell-free virus or 8×10^5 MT-2 cells) were pulsed to monocytes, but mature DCs differentiated from them did not stimulate autolo-

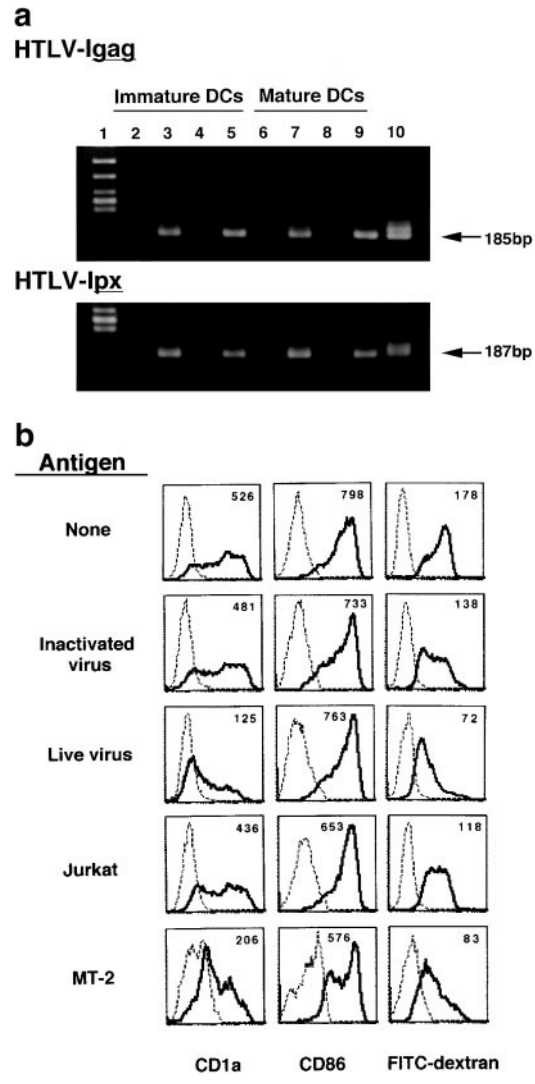

FIG. 5. (a) Detection of HTLV-I proviral DNA by nested PCR. Immature and mature DCs were generated from mock- or HTLV-I-infected monocytes. Monocytes were obtained from healthy donors and pulsed with 20 $CCID_{50}$ of HTLV-I or an equivalent dose of heat-inactivated virus. Genomic DNA was extracted from mock- or HTLV-I-infected immature or mature DCs and amplified by PCR. Lane 1: size marker. Lanes 2–5: immature DCs. Lane 2: case 1, mock-infected. Lane 3: case 1, HTLV-I-infected. Lane 4: case 2, mock-infected. Lane 5: case 2, HTLV-I-infected. Lanes 6–9: mature DCs. Lane 6: case 1, mock-infected. Lane 7: case 1, HTLV-I-infected. Lane 8: case 2, mock-infected. Lane 9: case 2, HTLV-I-infected. Lane 10: positive control. (b) Expression of various molecules and uptake of FITC–dextran by immature DCs generated from HTLV-I-infected monocytes. Monocytes were obtained from a healthy donor and pulsed with Ags. Twenty $CCID_{50}$ of HTLV-I or an equivalent dose of heat-inactivated virus and 2×10^5 cells/well of Jurkat or MT-2 cells were used as Ags. Immature DCs were generated from the Ag-pulsed monocytes by using rGM-CSF and rIL-4. The number in each panel represents the mean fluorescence intensity.

TABLE 1

Autologous T Cell Proliferative Responses to DCs from Monocytes Infected at Day 0 and Repulsed at Day 5 of the Immature DC Stage[a]

Antigen		Responder ([^{3}H]thymidine uptake; 10^3 cpm)		
Day 0 (Monocyte)	Day 5 (Immature DC)	Unseparated T cells	CD4$^+$ T cells	CD8$^+$ T cells
None	None	10.3 ± 0.7	16.7 ± 1.0	14.0 ± 1.1
Inactivated virus	Live virus	28.3 ± 0.9 *	49.7 ± 1.9 **	34.2 ± 2.3 **
Live virus	Live virus	5.9 ± 0.7 *	3.3 ± 0.6 **	4.4 ± 0.5 **
Jurkat	Live virus	56.5 ± 7.0 *	49.3 ± 2.2 *	30.3 ± 1.9 **
MT-2	Live virus	12.0 ± 0.8 *	13.0 ± 3.1 *	6.4 ± 1.2 **
None	Live virus	84.7 ± 5.6	42.9 ± 2.8	45.4 ± 2.6

[a] The responder T cells (1×10^5/cell) were stimulated for 5 days with autologous monocyte-derived mature DCs (5×10^3/well) obtained from a healthy donor. Monocytes were infected or remained uninfected on day 0 and were re-pulsed with HTLV-I (20 $CCID_{50}$) at day 5 of the immature DC state. The monocytes were infected by using 20 $CCID_{50}$ of cell-free HTLV-I or with 2×10^5/well of MT-2 cells. As a control, an identical amount of heat-inactivated HTLV-I or Jurkat cells was used. Representative results of three separate experiments are shown. Assays were done in triplicate, and the mean ± SD is shown. Statistically significant differences were calculated by using Student's *t* test.

* $P < 0.01$.

** $P < 0.001$.

gous T cells (not shown). In order to confirm the lack of APC function of DC from virus-infected monocytes, immature DCs from virus-infected monocytes were secondarily pulsed with cell-free HTLV-I on day 5 (Table 1). Positive control DCs (nonpulsed at the monocyte stage and pulsed at the immature DC stage with the cell-free virus) induced strong proliferation of T cells, and DCs from monocytes pulsed with inactivated virus or Jurkat cells on day 0 and secondarily pulsed with the HTLV-I at an immature DC stage induced almost the same level of proliferation of CD4$^+$ T cells and CD8$^+$ T cells, although the proliferation level of unseparated T cells seemed slightly reduced compared with that of positive control DCs. However, mature DCs differentiated from virus-infected monocytes did not stimulate unseparated T cells, CD4$^+$ T cells, or CD8$^+$ T cells, even when doubly infected with HTLV-I on day 5. There were statistical differences in the ability to stimulate T cells between DCs from monocytes pulsed with controls and those from HTLV-I-infected monocytes (Table 1). To test a different viral source, we established an HTLV-I-infected T cell line from a HAM/TSP patient and examined its influence on monocytes. Mature DCs generated from monocytes pulsed with the cells themselves or with cell-free virus obtained from them showed similar dysfunction (not shown).

Influence of HTLV-I infection of monocytes from healthy donors on IL-10 production

Since monocytes are known to produce IL-10 by various stimuli, we examined IL-10 production from monocytes that were donated by healthy donors and were infected with HTLV-I *in vitro* (Table 2). As a positive control, lipopolysaccharide (LPS) stimulation was used and the LPS-stimulated monocytes produced a high level of IL-10 24 h after stimulation. Monocytes infected with HTLV-I or pulsed with control Ags produced the cytokine on day 1; however, HTLV-I-infected monocytes secreted a significantly larger amount of IL-10 on day 3 after stimulation (20 $CCID_{50}$ cell-free virus and MT-2 cells; Table 2) than control Ag-pulsed monocytes. It might be important to determine whether IL-10 was exclusively

TABLE 2

Production of IL-10 by HTLV-I-Infected Monocytes[a]

Cell	Stimulation		IL-10 (pg/ml) Day 1	Day 3	
Monocytes	None		20.9 ± 6.3	8.9 ± 1.8	
Monocytes	LPS	10 ng/ml	728.2 ± 3.5	92.0 ± 3.5	
Monocytes	Inactivated HTLV-I	10 $CCID_{50}$	273.3 ± 27.3	50.8 ± 3.3	
		20 $CCID_{50}$	190.2 ± 25.0	37.3 ± 0.8	
Monocytes	Live HTLV-I	10 $CCID_{50}$	172.4 ± 6.9	72.9 ± 8.3	*
		20 $CCID_{50}$	178.2 ± 9.7	71.8 ± 2.6	
Monocytes	Jurkat	0.3×10^5	99.6 ± 14.5	30.0 ± 5.1	
		1.0×10^5	197.3 ± 14.7	32.7 ± 2.4	**
	MT-2	0.3×10^5	395.5 ± 29.4	83.5 ± 14.5	
		1.0×10^5	579.0 ± 10.7	85.3 ± 3.8	*
Jurkat	None		0	0	
MT-2	None		2.1 ± 0.6	1.4 ± 0.9	

[a] Purified monocytes were obtained from a healthy donor, and IL-10 secreted into the supernatant of monocytes (5×10^6/ml) cultured in the presence of various Ags was measured. The supernatant was harvested on days 1 and 3 after the culture. Representative results of three separate experiments are shown. Assays were done in triplicate, and the mean ± SD is shown. Statistically significant differences were calculated by using Student's *t* test.

* $P < 0.01$.

** $P < 0.05$.

produced by HTLV-I-infected monocytes. However, we could not discriminate viral-infected monocytes from control Ag-pulsed monocytes at the single-cell level.

DISCUSSION

In this study, we successfully differentiated monocyte-derived DCs from approximately 70% of acute-type ATL patients; however, the production rate of DCs from patients' monocytes was reduced (not shown), allowing us to conduct only selected, but not full, experiments. Previously we showed that the DCs from HAM/TSP patients rapidly differentiated to both immature and mature DCs and extensively stimulated autologous $CD4^+$ and $CD8^+$ T cells *in vitro* (Makino *et al.,* 1999). In contrast to the DCs from HAM/TSP patients, the expression of CD1a and CD86 Ags and the internalization activity of exogenous Ags through the mannose receptor were deteriorated in immature DCs obtained from ATL patients. The APC function of mature DCs from ATL patients was determined by using allogeneic MLR because of immunodeficiency of patients' T cells (Uchiyama *et al.,* 1977). The allogeneic stimulus is strong in general, and the T cell proliferation response might be less affected by changes in the DC phenotype (Takahashi *et al.,* 1998). Therefore, the APC function of patients' mature DCs could not be evaluated correctly in this study. However, elevated stimulating activity as seen in HAM/TSP patients was not observed in the least; rather the expression of CD40, CD86, or MHC class II Ags was reduced depending on the case (Fig. 1b). Generally, functional deficiency of DCs was observed in tumor-bearing animal models (Chaux *et al.,* 1997; Ishida *et al.,* 1998). In these mice, monocytes could not differentiate properly to DCs and, in some cases, some cytokines or factors have been reported to induce the defect of their maturation (Allavena *et al.,* 1998; Caux *et al.,* 1994; Ishida *et al.,* 1998; Macatonia *et al.,* 1993). It has not been apparent whether functional abnormalities of DCs observed in ATL patients are induced primarily by HTLV-I infection or secondarily by HTLV-I-infected activated T cells. However, monocytes are known to be susceptible to HTLV-I infection *in vitro* and *in vivo* (Koyanagi *et al.,* 1993; Wakamatsu *et al.,* 1999) and monocytes from ATL patients were more strongly down-regulated in CD14 Ag expression after adhesion to plastic in the absence of T cells. Furthermore, no DCs were produced in around 30% of the patients examined (3/11). Therefore, it is possible that the maturation defect of DCs occurs in ATL patients as in tumor-bearing animals. In order to clarify this point, we tried to differentiate DCs from HTLV-I-infected monocytes.

The monocytes donated from healthy individuals were easily infected with HTLV-I *in vitro* as evidenced by surface expression of HTLV-I*gag* Ags and detection of proviral DNA from day 1 to day 5 after infection. Interestingly the *in vitro* infected monocytes were down-regulated in the expression of CD14 molecules. The lack of CD14 expression is reported to be closely associated with induction of apoptotic cell death of monocytes (Devitt *et al.,* 1998; Heidenreich *et al.,* 1997). However, despite incompetent CD14 expression, HTLV-I-infected monocytes convincingly stimulated allogeneic T cells, expressed receptors for GM-CSF and IL-4, and differentiated into DCs without showing apoptosis (not shown). We could not determine quantitatively the proviral copy number in DCs differentiated from HTLV-I-infected monocytes because of nested PCR. Furthermore, the viral infection rate of the DCs seemed to be very low because the intensity of bands from the DCs was obviously lower than that from an identical number of TL-Om1 cells, which carry a single proviral DNA copy. Immature DCs generated from the virus-infected monocytes were, however, moderately suppressed in the expression of CD1a Ag and in the internalization activity of FITC–dextran. Therefore, although more detailed functional analyses of immature DCs remain to be conducted by using superantigens and intracellular pathogens, the most important function of the immature DCs seems to be at least deteriorated. Mature DCs are able to stimulate Ag-specific T cells. Their T-cell-stimulating function has been reported to be associated with high-level expression of MHC, adhesion, and costimulatory molecules (Sallusto and Lanzaveccia, 1994; Scheinecker *et al.,* 1998; Young and Inaba, 1996). Although DCs generated from HTLV-I-infected monocytes rather competently expressed those molecules (Fig. 1b) and were infected with HTLV-I, they could not stimulate autologous T cells. This point was confirmed by double-pulse experiments. Mature DCs generated after being doubly infected on day 0 in the monocyte stage and on day 5 in the immature DC stage still did not stimulate autologous $CD4^+$ T cells or $CD8^+$ T cells. In addition to the MHC, adhesion, and costimulatory molecules, it has been reported that molecules on DCs such as a C-C chemokine termed DC-CK1 (Adema *et al.,* 1997) and a receptor for activator of NF-κB (RANK) (Anderson *et al.,* 1997) play important roles in conduction of APC function. Further work will be required to clarify the relationship between these molecules and the HTLV-I-infected monocyte-derived DCs. However, aside from these molecules, the failure of doubly pulsed DCs to stimulate autologous T cells might be simply due to reduced internalization activity at the immature stage.

In addition to T-cell-stimulating DCs, tolerogenic DCs are known (Finkelman *et al.,* 1996; Steptoe and Thomson, 1996). It might be possible that DCs generated from virus-infected monocytes acted tolerogenically. The tolerogenic DCs have been produced by various experimental procedures, and they induce T cell tolerance against the exposed Ag (Ruedl and Hubele, 1997; Viney *et al.,* 1998). However, in general, these DCs are remarkably down-regulated in the expression of costimulatory molecules such as CD80 and CD86. In this respect, the

mature DCs generated by HTLV-I-infected monocytes, in which the expression of CD86 Ags was not reduced, seem phenotypically different from the tolerogenic DCs (not shown).

In many tumor-bearing animal models, the functional deficiency of DCs was observed. In these animals, maturation defects are commonly observed and IL-10 and vascular endothelial growth factor (VEGF) are reported to be some of their important inducers (Allavena *et al.,* 1998; Caux *et al.,* 1994; Gabriovich *et al.,* 1996; Heidenreich *et al.,* 1997; Ishida *et al.,* 1998). Although we could not assess the role of VEGF, prolonged production of IL-10 by HTLV-I-infected monocytes was observed. In particular, IL-10 is reported to exhibit DC inhibition activity at a relatively late stage of DC maturation (Ishida *et al.,* 1998). Therefore, the significantly higher production of the cytokine at day 3 of culture of infected monocytes might be associated with the maturation defects and production of dysfunctional DCs.

It is extremely important to decide whether the functional defect of DC observed in this study is induced primarily, that is, preceding abnormal T cell expansion, or induced secondarily due to T cell leukemogenesis, especially since we would like to develop an immunotherapeutic strategy against ATL. However, in ATL patients, both DC dysfunction and T cell abnormalities were commonly observed. Therefore it is not easy to address this important point by studies using PBMCs from ATL patients, but the functional analyses on PBMCs from asymptomatic carriers will be helpful in finding an answer to this question.

In conclusion, DCs obtained from HTLV-I-infected monocytes did not fully take up exogenous Ags and did not stimulate autologous T cells. Therefore, they might be closely associated with the development of immunodeficiency. Although DCs obtained from ATL patients were not identical to the HTLV-I-infected monocyte-derived DCs, especially in terms of phenotypic characteristics, the latter type of DC could be a potent model capable of at least partially clarifying the immunological mechanism leading to DC deficiency in ATL patients.

MATERIALS AND METHODS

Study subjects and cell preparation

PBMCs were donated under informed consent by 11 patients diagnosed as acute-type ATL at Kagoshima University Hospital and by 10 uninfected healthy individuals. The ATL patients were not undergoing treatment with any immunosuppressants or glucocorticoid steroid hormones. The PBMCs were isolated from heparinized blood by using Ficoll–Paque (Pharmacia, Uppsala, Sweden) and were cryopreserved in liquid nitrogen until used as described (Makino and Baba, 1997). Plastic-adherent monocytes were isolated from either freshly isolated or cryopreserved PBMCs, and DCs were differentiated by culturing them with 1% autologous plasma for 5 days in the presence of 50 ng of recombinant (r)GM-CSF (donated by Kirin Brewery Co., Tokyo, Japan) and 10 ng of rIL-4 (Genzyme, Cambridge, MA) per milliliter. rGM-CSF and rIL-4 were supplied every 2 days as described previously (Makino and Baba, 1997). On day 0 of monocyte culture, they were pulsed with cell-free HTLV-I, heat-inactivated (56°C for 30 min) virus, a HTLV-I-producing cell line MT-2, or a control lymphoma cell line, Jurkat. Before being pulsed, both MT-2 and Jurkat cells were treated with 200 μg/ml of mitomycin C for 30 min at 37°C. In some cases, immature DCs were pulsed with the cell-free HTLV-I on day 5 of cell culture. Maturation of DCs was accomplished by treating them with 10 ng of LPS (*Escherichia coli* 0111: B4, Difco Laboratories, Detroit, MI) per milliliter for 24 h and mature DCs were assessed for APC function after treatment with 50 μg/ml of mitomycin C. The mitomycin C-treated MT-2 and Jurkat cells were removed from live DC populations by using 30–55% gradient Percoll solutions. Less than 3% of MT-2 or Jurkat cells might be contaminated in the DC population. DCs were also generated from peripheral monocytes of ATL patients. In this case, the contamination of the monocyte population with HTLV-I-infected cells was avoided by removing $CD2^+$ T cells from PBMCs in advance by using immunomagnetic beads coated with anti-CD2 monoclonal Abs (mAbs) (Dynabeads 450, Dynal, Oslo, Norway), and subsequently monocytes were prepared and DCs were differentiated. More than 98% of the $CD2^+$ T cells were depleted from PBMCs. The concentration of IL-10 secreted into culture supernatant fluids of LPS-stimulated or Ag-pulsed monocytes (5×10^6/ml) was measured by using an ELISA kit (Genzyme Diagnostic, Cambridge, MA) according to the method described by Foey *et al.* (1998).

Preparation of cell-free HTLV-I virions and virus-producing cell line

An HTLV-I-producing cell line, MT-2, was generously given to us by Dr. I. Miyoshi, Kochi Medical School, and cell-free HTLV-I virions were prepared by sonicating 5×10^6 MT-2 cells per milliliter for 50 s. Both virions released into the supernatant from the sonicated MT-2 cells and MT-2 cells themselves were used as a viral source. For detection of HTLV-I proviral DNA, DNA was prepared from monocytes, DCs, or $CD4^+$ T cells by using a DNA extractor kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The DNA was amplified by PCR using primer pairs corresponding to nucleotides 1729 to 1750 and 1942 to 1964 of the HTLV-I*gag* region or to nucleotides 6496 to 6518 and 6696 to 6718 of the HTLV-I*px* region. When no band or only a faint band was detected in the PCR products, nested PCR was conducted by using a primer pair corresponding to nucleotides 1756 to 1777 and 1919 to 1940 of the HTLV-I*gag* region or to

nucleotides 6509 to 6531 and 6673 to 6695 of the HTLV-I*px* region. The HTLV-I*gag* and *px* proviral DNAs were detected as 185- and 187-bp bands. The $CCID_{50}$ of cell-free HTLV-I was determined by using $CD4^+$ T cells in the presence of 2 μg/ml of phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI) and 100 U/ml of rIL-2 (TGP-3, Takeda Chemical Industries, Osaka, Japan). Monocytes and DCs were infected with 20 $CCID_{50}$ of HTLV-I. Infection of monocytes and DCs with HTLV-I was also determined by cytometry analysis (FACScan, Becton Dickinson Immunocytometry Systems, San Jose, CA) for surface expression of HTLV-I*gag* Ags. In some cases, $CD4^+$ T cells obtained from uninfected donors were infected by using MT-2 as described previously (Takamoto *et al.,* 1997) and were used as a viral source.

Analysis of cell surface Ag

The expression of cell surface Ag on monocytes, immature DCs, and mature DCs was determined by using FACScan. Live cells (1×10^4) were gated and analyzed. To eliminate dead cells from analysis, propidium iodide (Sigma Chemical Co., St. Louis, MO) was used. We used FITC-conjugated mAbs against human HLA-ABC (G46-2.6) and HLA-DR (L243, Becton Dickinson), CD40 (5C3, PharMingen, San Diego, CA), phycoerythrin-labeled mAbs to CD54 (HA58), CD58 (L304.4) (Becton Dickinson), CD86 (IT2.2, PharMingen), and CD83 (HB15a, Immunotech), and purified murine mAbs to CD1a (NA1/34, Serotec Ltd., Oxford, England), HTLV-I*gag* (GIN14), and HTLV-I*env* (F10) (generously given to us by Dr. Y. Tanaka, Department of Hygiene, Kitazato University, Sagamihara, Japan), which were followed by FITC-labeled goat $F(ab')_2$ anti-mouse immunoglobulins (Igs) (Tagoimmunologicals, Camarillo, CA). The optimal concentrations of mAbs were determined in advance. Monocytes and DCs under an apoptotic process were determined by staining them with FITC-conjugated Annexin V (Genzyme). The ability of immature DCs to internalize FITC–dextran (MW 42,000, Sigma) through a mannose receptor was tested. In brief, DCs were harvested and resuspended at 10^6 cells per milliliter in RPMI medium supplemented with 10% fetal calf serum (FCS) and HEPES (pH 7.4). Two hundred microliters of DC suspension was preincubated for 3 min at 37°C and 20 μl of a 1 mg/ml FITC–dextran solution was added. At 30 min of incubation, the reaction was stopped by addition of 4 ml of cold PBS supplemented with 0.2% bovine serum albumin, 0.01% NaN_3, and 2 mM EDTA, and the cells were washed four times at 4°C in the same buffer. The uptake of FITC–dextran by propidium iodide-gated viable cells was analyzed using FACScan.

Proliferation assay

Autologous and/or allogeneic DC-T cell mixed lymphocyte cultures were conducted by using DCs from ATL patients and those differentiated from HTLV-I-infected monocytes. The DCs were harvested on day 7 of HTLV-I infection as stimulator cells. Unseparated T cells, purified $CD4^+$ T cells, or $CD8^+$ T cells were used as a responder population. DCs that were unpulsed, infected by using cell-free HTLV-I or MT-2 cells, or pulsed with heat-inactivated HTLV-I Ag or Jurkat cells were treated with mitomycin C and plated to give a DC to responder cell ratio of 20 or 40. Proliferation of stimulator cells was confirmed to be completely blocked by mitomycin C. The responder T cells were purified as follows: freshly thawed PBMCs were depleted of MHC class II^+ cells by using magnetic beads coated with mAbs to MHC class II Ags (Dynabeads 450, Dynal) and further treated with beads coated with either CD4 or CD8 mAbs to select T cells negatively. The purity of $CD4^+$ T cells or $CD8^+$ T cells was more than 98% and the contamination of the purified population by HLA-DR-positive cells was less than 2%. Responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates. The cell proliferation during the last 16 h of a 5- to 6-day culture in the presence of 10% heat-inactivated human serum (a generous gift from the Kagoshima Red Cross Blood Center) was quantified by incubating the cells with 1 μCi/well of [^{3}H]thymidine. The results are expressed as the mean difference in counts per minute obtained from triplicate cultures.

ACKNOWLEDGMENTS

We acknowledge the contribution of Ms. N. Makino to the preparation of the manuscript and we thank Dr. M. L. Robbins for reviewing the manuscript. This work was supported in part by a Grant-in-Aid for a Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan.

REFERENCES

Adema, G. J., Hartgers, F., Verstraten, R., de Vries, E., Marland, G., Menon, S., Foster, J., Xu, Y., Nooyen, P., McClanahan, T., Bacon, K. B., and Figdor, C. G. (1997). A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* **387,** 713–717.

Allavena, P., Piemonti, L., Longoni, D., Bernasconi, S., Stoppacciaro, A., Ruco, L., and Mantovani, A. (1998). IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. *Eur. J. Immunol.* **28,** 359–369.

Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Teepe, M. C., Dubose, R. F., Cosman, D., and Galibert, L. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390,** 175–179.

Caux, C., Massacrier, C., Vanbervliet, B., Barthelemy, C., Kiu, Y. J., and Banchereau, J. (1994). Interleukin 10 inhibits T cell alloreaction induced by human dendritic cells. *Int. Immunol.* **6,** 1177–1185.

Chaux, P., Favre, N., Martin, M., and Martin, F. (1997). Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. *Int. J. Cancer* **72,** 619–624.

Devitt, A., Moffatt, O. D., Raykundalia, C., Capra, J. D., Simmons, D. L., and Gregory, C. D. (1998). Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* **392,** 505–509.

Enk, A. H., Angeloni, V. L., Udey, M. C., and Katz, S. I. (1993). Inhibition of Langerhans cell antigen-presenting function by IL-10: A role for IL-10 in induction of tolerance. *J. Immunol.* **151,** 2390–2398.

Finkelman, F. D., Lees, A., Birnbaum, R., Gause, W. C., and Morris, S. C. (1996). Dendritic cells can present antigen in vivo in a tolerogenic or immunogenic fashion. *J. Immunol.* **157,** 1406–1414.

Foey, A. D., Parry, S. L., Williams, L. M., Feldmann, M., Foxwell, B. M. J., and Brennan, F. M. (1998). Regulation of monocyte IL-10 synthesis by endogenous IL-1 and TNF-α: Role of the p38 and p42/44 mitogen-activated protein kinases. *J. Immunol.* **160,** 920–928.

Gabriovich, D. I., Chen, H. L., Girgis, K. R., Thomas Cunningham, H., Meny, G. M., Nadaf, S., Kavanaugh, D., and Carbone, D. P. (1996). Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.* **2,** 1096–1103.

Heidenreich, S., Schmidt, M., August, C., Cullen, P., Rademaekers, A., and Pauels, H-G. (1997). Regulation of human monocyte apoptosis by the CD14 molecule. *J. Immunol.* **159,** 3178–3188.

Hollsberg, P., and Hafler, D. A. (1993). Pathogenesis of diseases induced by human lymphotropic virus type I infection. *N. Engl. J. Med.* **328,** 1173–1182.

Inaba, K., Metley, J. P., Crowley, M. T., and Steinman, R. M. (1990). Dendritic cells pulsed with protein antigen in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J. Exp. Med.* **172,** 631–640.

Inaba, K., Young, J. W., and Steinman, R. M. (1987). Direct activation of $CD8^+$ cytotoxic T lymphocytes by dendritic cells. *J. Exp. Med.* **166,** 182–194.

Ishida, T., Oyama, T., Carbone, D. P., and Gabrilovich, D. I. (1998). Defective function of Langerhans cells in tumor-bearing animals is the result of defective maturation from hemopoietic progenitors. *J. Immunol.* **161,** 4842–4851.

Kannagi, M., Harada, S., Maruyama, I., Inoko, H., Igarashi, H., Kuwashima, G., Sato, S., Morita, M., Kidokoro, M., Sugimoto, M., Funahashi, S., Osame, M., and Shida, H. (1991). Predominant recognition of human T cell leukemia virus type I (HTLV-I) px gene products by human $CD8^+$ cytotoxic T cells directed against HTLV-I-infected cells. *Int. Immunol.* **3,** 761–767.

Kannagi, M., Sugamura, K., Sato, H., Okochi, K., Uchino, H., and Hinuma, Y. (1983). Establishment of human cytotoxic T cell lines specific for human adult T cell leukemia virus-bearing cells. *J. Immunol.* **130,** 2942–2946.

Koyanagi, Y., Itoyama, Y., Nakamura, N., Takamatsu, K., Kira, J., Iwamasa, T., Goto, I., and Yamamoto, N. (1993). In vivo infection of human T-cell leukemia virus type I in non-T cells. *Virology* **196,** 25–33.

Macatonia, S. E., Doherty, T. M., Knight, S. C., and O'Garra, A. (1993). Differential effect of IL-10 on dendritic cell-induced T cell proliferation and IFN-γ production. *J. Immunol.* **150,** 3755–3765.

Makino, M., and Baba, M. (1997). Establishment of a cryopreservation method of human peripheral blood mononuclear cells for efficient production of dendritic cells. *Scand. J. Immunol.* **45,** 618–622.

Makino, M., Shimokubo, S., Wakamatsu, S., Izumo, S., and Baba, M. (1999). The role of HTLV-I-infected dendritic cells in the development of HTLV-I-associated myelopathy/tropical spastic paraparesis. *J. Virol.* **73,** 4575–4581.

O'Doherty, U., Steinman, R. M., Peng, M., Cameron, P. U., Gezelter, S., Kopeloff, I., Swiggard, W. J., Pope, M., and Bhardwaj, N. (1993). Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J. Exp. Med.* **178,** 1067–1078.

Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., Matsumoto, M., and Tara, M. (1986). HTLV-I associated myelopathy, a new clinical entity. *Lancet* **i,** 1031–1032.

Reis e Sousa, C., Stahl, P. D., and Austyn, J. M. (1993). Phagocytosis of antigens by Langerhans cells in vitro. *J. Exp. Med.* **178,** 509–519.

Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F., and Austyn, J. M. (1995). Dendritic cell loss from non-lymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J. Exp. Med.* **181,** 2237–2247.

Ruedl, C., and Hubele, S. (1997). Maturation of Peyer's patch dendritic cells in vitro upon stimulation via cytokines or CD40 triggering. *Eur. J. Immunol.* **27,** 1325–1330.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* **179,** 1109–1118.

Scheinecker, C., Machold, K. P., Majdic, O., Höcker, P., Knapp, W., and Smolen, J. S. (1998). Initiation of the autologous mixed lymphocyte reaction requires the expression of costimulatory molecules B7–1 and B7–2 on human peripheral blood dendritic cells. *J. Immunol.* **161,** 3966–3973.

Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J., and Enk, A. H. (1997). Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* **159,** 4772–4780.

Steptoe, R. J., and Thomson, A. W. (1996). Dendritic cells and tolerance induction. *Clin. Exp. Immunol.* **105,** 397–402.

Takahashi, K., Honeyman, M. C., and Harrison, L. C. (1998). Impaired yield, phenotype, and function of monocyte-derived dendritic cells in humans at risk for insulin-dependent diabetes. *J. Immunol.* **161,** 2629–2635.

Takamoto, T., Makino, M., Azuma, M., Tanaka, A., Kanzaki, T., Baba, M., and Sonoda, S. (1997). HTLV-I-infected T cells activate autologous $CD4^+$ T cells susceptible to HTLV-I infection in a costimulatory molecule-dependent fashion. *Eur. J. Immunol.* **27,** 1427–1432.

Takatsuki, K., Uchiyama, T., Sagawa, K., and Yodoi, J. (1977). Adult T cell leukemia in Japan. *In* "Topics in Hematology" (S. Seno, F. Takaku, and S. Irino, Eds.), pp. 73–77. Excerota Medica, Amsterdam.

Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K., and Uchino, H. (1977). Adult T-cell leukemia: Clinical and hematologic features of 16 cases. *Blood* **50,** 481–492.

Viney, J. L., Mowat, A. M., O'Malley, J. M., Williamson, E., and Fanger, N. A. (1998). Expanding dendritic cells in vivo enhances the induction of oral tolerance. *J. Immunol.* **160,** 5815–5825.

Wakamatsu, S., Makino, M., Tei, C., and Baba, M. (1999). Monocyte-driven activation-induced apoptotic cell death of human T-lymphotropic virus type I-infected T cells. *J. Immunol.* **163,** 3914–3919.

Young, J. W., and Inaba, K. (1996). Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J. Exp. Med.* **183,** 7–11.