Characterization of Monocyte-Derived Dendritic Cells from Cats Infected with Feline Immunodeficiency Virus

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ABSTRACT. Dendritic cells (DCs) are professional antigen presenting cells (APCs) that possess an extraordinary capacity to stimulate naïve T cells and initiate a primary immune response. To develop a DC-based immunotherapy for feline immunodeficiency virus (FIV) infection, we carried out a study to characterize DCs from FIV-infected cats and compared the observations with those obtained from healthy controls. DCs were derived from adherent peripheral blood mononuclear cells that had been cultivated with recombinant feline interleukin 4, granulocyte macrophage colony-stimulating factor and heat-inactivated autologous plasma. Various parameters, such as cell morphology, surface phenotype, endocytosis and mixed leukocyte reaction (MLR), were analyzed to characterize feline DCs. Monocyte-derived DCs from FIV-infected cats as well as those from healthy controls showed a dendritic appearance and expressed an APC-like phenotype (CD1c+, CD80+ and MHC class II+). However, the expression level of CD1a was variable in the DCs derived from FIV-infected cats, although this was not the case in the DCs derived from the healthy controls. DCs from the FIV-infected cats retained the ability to take up dextran via the mannose receptor and also showed an apparent MLR, indicating that these cells could be useful in immunotherapy. In this study, monocytes obtained from FIV-infected cats could differentiate into functional DCs, suggesting that they might be used in a DC-based immunotherapy against FIV infection.

KEY WORDS: Dendritic cell, feline immunodeficiency virus, immunotherapy.

FULL PAPER


Dendritic cells (DCs), first characterized in 1973 [28], are recognized as professional antigen presenting cells (APCs) that possess the unique ability of priming naïve T cells. DCs are derived from hematopoietic stem cells. After immature DCs take up and process antigens in the peripheral tissues, they differentiate into mature DCs and migrate to draining lymph nodes where they present antigens and stimulate naïve T cells to induce antigen-specific immunity. Thus, DCs play a pivotal role in the immune system by acting as “sentinel cells” [2].

In the 1990s, a method for differentiating peripheral precursor cells into DCs was established in mice and humans [17, 25]. Since then, there has been rapid progress in studies involving DCs. Monocytes incubated with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) acquire the properties of DCs. Moreover, several studies have been performed to establish a method for generating feline DCs [4, 13, 27]. Similar to human and murine DCs, feline DCs have been generated from monocytes cultured with GM-CSF and IL-4, and they also possessed the characteristic properties of DCs.

Since DCs are known to be the most potent APCs, the benefits of a DC-based immunotherapy have been investigated in a variety of diseases, including cancer [12], autoimmune diseases [29], transplant rejection [16], allergic diseases [1] and infectious diseases [7]. In previous studies of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections, monocyte-derived DCs pulsed with inactivated viruses could induce a remarkable enhancement in both cellular and humoral immune responses [20, 21]. Hence, DC-based immunotherapy is expected to be a potential therapeutic candidate for HIV infection.

Feline immunodeficiency virus (FIV) infection is known to be associated with development of immunodeficiency in domestic cats that is similar to that in humans infected with HIV [3]. The mechanism of immunodeficiency development is associated with lymphoid depletion in the lymph nodes [22] and subsequent lymphopenia [15]. To date, no effective antiviral therapy has been applied to clinical use in cats naturally infected with FIV.

To assess the potential of utilizing an autologous DC-based immunotherapy in treatment of cats infected with FIV, we investigated several immunological properties of monocyte-derived DCs from FIV-infected cats in comparison to those from healthy controls.

MATERIALS AND METHODS

Cats and blood samples: Seven FIV-infected cats referred to the Veterinary Medical Center of the University of Tokyo were used in this study. All cats were shown to be positive for FIV antibody and negative for feline leukemia virus
(FeLV) antigen by a commercial ELISA test kit (IDEXX Laboratories, Portland, OR, U.S.A.). These cats showed no clinical signs associated with FIV infection and were categorized into the asymptomatic carrier stage. Eighteen cats that were seronegative for FIV antibody and FeLV antigen were used as the healthy controls. Autologous feline plasma, obtained from each cat, was inactivated by heating at 56°C for 45 min and used as a supplement for the culture medium.

**Cell preparation**: The methods for the preparation of DCs and macrophages were modified based on previously described protocols [4, 13, 27]. Briefly, heparinized whole blood was layered onto Ficoll/Hypaque (Lymphoprep; Nycomed Pharma AS, Oslo, Norway) and centrifuged at 560 g for 45 min at room temperature. Peripheral blood mononuclear cells (PBMCs) at the interface were harvested and washed twice with PBS (Sigma, St. Louis, MO, U.S.A.). To remove the contaminating platelets and red blood cells, PBMCs were incubated in ammonium chloride at 56°C for 45 min and used as a supplement for the culture medium.

They were then resuspended in RPMI-1640 supplemented with 100 U of penicillin per mL, 0.1 mg of streptomycin per mL, and 2 mM glutamine. These cells were then incubated in tissue culture flasks at 37°C with 5% CO2 (day 1). After 24 hr (day 2), the non-adherent cells were removed by washing with RPMI-1640. The adherent cells were maintained in the culture medium containing 10% heat-inactivated autologous plasma. For the induction of DCs, 100 ng of recombinant feline (r-fe) GM-CSF per mL and 50 ng of r-fe IL-4 (R&D Systems, Minneapolis, MN, U.S.A.) per mL were added to the culture medium, but not for the macrophages cultures. The medium was refreshed on day 5 of culture. The adherent cultured cells were harvested on either day 7 or day 8. For maturation of DCs and macrophages, the cells were stimulated with 10 μg of LPS (Sigma) per mL during the last 24 hr.

**Flow cytometry (FCM) analysis and antibodies**: The expression of surface markers on DCs and macrophages was analyzed by FCM. The cells were detached from the flask by trypsinization and resuspended in FCM buffer (PBS containing 2% [v/v] fetal calf serum [FCS]). This FCM buffer was used throughout the staining procedure. The cells were always kept on ice or at 4°C. They were stained at 4°C for 30 min with antibodies that recognize the cell surface markers or isotype-matched antibodies. The cells were then washed in FCM buffer and resuspended in FITC-conjugated anti-mouse IgG (Sero Tech Ltd., Oxford, UK). After another wash, the cells were analyzed for surface expression using a Becton Dickinson FACSCalibur flow cytometer and the CellQuest software (Becton Dickinson, Mountain View, CA, U.S.A.).

The isotype-matched control antibodies, murine IgG1k (MOPC-31C, BD Pharmingen, San Jose, CA, U.S.A.) or murine IgG2B (133303, R&D Systems), were used to determine background staining and autofluorescence. The mAbs used in this study were FE1.5F4 for CD1a, FE5.5C1 for CD1c, FE1.7B12 for CD4, FE1.1B11 for CD5, CA16.3E10 for CD11b, FE3.9F2 for CD18, CA2.1D6 for CD21 and 42.3 for MHC class II (all the above were obtained from the Leukocyte Antigen Biology Laboratory, Davis, CA, U.S.A.). Furthermore, B7.1 66 was used for CD80 [30], 44717 was used for CXCR4 (R&D Systems), and 9F23 was used for IL-2 receptor α-chain (IL-2Rα) [24].

For detection of FIV p24, DCs and macrophages were fixed and permeabilized with 4% paraformaldehyde and 0.1% saponin (Cytofix/Cytoperm Buffer, BD Pharmingen) for 15 min. The cells were then washed with PBS containing 2% FCS and 0.1% saponin (Perm/Wash Buffer, BD Pharmingen) and reacted on ice for 30 min with anti-FIV p24 mAb (Biogenesis, Hackensack, NJ) or the isotype-matched control antibody, murine IgG1k (MOPC-31C, BD Pharmingen). The cells were analyzed by FCM after being washed with Perm/Wash Buffer and reacted with FITC-conjugated mouse IgG (Sero Tech Ltd., Oxford, UK).

**Endocytosis assay**: Non-stimulated immature cells were washed with PBS and incubated in RPMI-1640 with 0.1 mg of FITC-dextran (DX-FITC, molecular weight 40,000; Molecular Probes, Carlsbad, CA, U.S.A.) per mL at 37°C for 1 hr. Nonspecific DX-FITC binding to the cell surface was assessed by incubating on ice. In order to inhibit the mannose receptor, 5 mg of mannan (Sigma) per mL was added to the solution beforehand. DX-FITC uptake was terminated by adding ice-cold FCM buffer. The cells were then washed extensively with FCM buffer, and DX-FITC uptake was quantified by measuring the fluorescence intensity using a Becton Dickinson FACSCalibur flow cytometer and the CellQuest software (Becton Dickinson).

**Mixed leukocyte reaction (MLR)**: Cultured cells used as stimulator cells were treated with 0.05 mg of mitomycin C (Sigma) per mL at 37°C for 30 min, and then washed twice with PBS. Feline allogeneic PBMCs (5 × 10^4 cells) were cocultured at different stimulator cell to effector cell ratios for 3 days. Cell proliferation was measured by determining BrdU incorporation during DNA synthesis using the Cell Proliferation ELISA, BrdU colorimetric kit (Roche, Indianapolis, IN, U.S.A.), according to the manufacturer’s instructions. The results were expressed in terms of the ELISA value, which was the difference in the absorbance between the co-cultured and cytokine monocolulated APC experiments.

**RESULTS**

**Morphology of DCs**: Adherent PBMCs from FIV-infected cats were cultured in the presence or absence of GM-CSF and IL-4. During the culture period, most of the cells were round in shape; however, a few cells showed a dendritic appearance. After the LPS stimulus during the last 24 hr, most of the cells cultured with cytokines remained adherent and were large and irregularly shaped with long processes or veils extending from the cell body; this appearance is typical of mature dendritic cells (Fig. 1a). On the other hand, cells cultured without cytokines exhibited a typical macrophage-like morphology that is characterized by...
the presence of numerous vacuoles in the cytoplasm (Fig. 1c). Monocytes obtained from healthy controls showed essentially the same results (Fig. 1b and d).

**Surface phenotype of DCs:** The surface phenotypes of adherent PBMCs cultured with cytokines (IL-4 and GM-CSF) and LPS were analyzed (Fig. 2). Expression of CD1c, CD80, MHC class II and CXCR4 was detectable on DCs from both FIV-infected cats and healthy controls (Fig. 2a). CD18 and IL-2R were also expressed on DCs from both FIV-infected cats and healthy controls (data not shown); however, CD4, CD5 and CD21 (T cell and B cell markers) were not expressed (data not shown). Expression of CD11b was detectable on DCs obtained from all the examined FIV-infected cats but was not detectable on those from healthy controls (Fig. 2a). Expression of CD1a was detected on DCs derived from all the examined FIV-infected cats but was not detectable on those from healthy controls (Fig. 2b). However, the expression was variable on DCs derived from the FIV-infected cats (Fig. 2b). CD1a was expressed on DCs from five of the seven FIV-infected cats (case 1 in Fig. 2b is shown as representative data), whereas its expression was low on the DCs from the remaining two FIV-infected cats (cases 2 and 3 in Fig. 2b). Although there was a slight difference between the FIV-infected cats and healthy controls, the cells expressed an APC-like phenotype. Moreover, expression of p24, which is an FIV-specific antigen, was not detected in the DCs derived from the FIV-infected cats (data not shown).

**Antigen uptake by DCs:** To investigate ability to take up exogenous antigens, monocyte-derived DCs and macrophages were incubated with DX-FITC and subsequently analyzed by FCM. DCs derived from the FIV-infected cats could take up dextran (Fig. 3a). DX-FITC uptake was inhibited when these cells were preincubated with mannan, which inhibits antigen uptake capacity via the mannose receptor (Fig. 3a). DCs derived from the uninfected cats showed essentially the same results (Fig. 3b). The amounts of DX-FITC uptake in macrophages derived from both the FIV-infected and uninfected cats were minimal (Fig. 3c and 3d).

**Stimulatory capacity of DCs:** To evaluate the immunostimulatory capacities of monocyte-derived DCs and macrophages, an allogeneic MLR was carried out. DCs and macrophages derived from the FIV-infected cats could generate proliferation of MHC-mismatched responder lympho-
cytes (Fig. 4a). These effects were dependent on the stimulator cell to responder cell ratio. The proliferative responses of the allogeneic feline PBMCs after co-culture with DCs and macrophages derived from the uninfected cats were similar to those after co-culture with DCs and macrophages derived from the FIV-infected cats (Fig. 4b).

DISCUSSION

The purpose of this study was to obtain basic information on DCs derived from FIV-infected cats for the development of a DC-based immunotherapy against FIV infection. The adherent PBMCs derived from the FIV-infected cats could differentiate into DCs when they were cultured with IL-4 and GM-CSF and subsequently stimulated with LPS. DCs derived from the FIV-infected cats as well as the healthy controls showed a dendritic appearance and expressed surface molecules that were characteristic of APCs (CD1c, CD80, MHC class II).

The CD1 family has evolutionarily diverged from the MHC class I and class II molecules and is associated with antigen presentation in association with lipids and glycolipids [8]. CD1a, CD1b and CD1c are also known to be expressed on feline DCs [4]. In the present study, the expression levels of CD1a in the DCs derived from 2 of the 7 FIV-infected cats were lower than those of the DCs derived from the uninfected cats. This finding was in good agreement with those of previous reports showing that DCs from HIV-infected patients expressed lower levels of CD1a compared with DCs from healthy volunteer donors [26]. Considering that CD1a is one of the maturation markers on DCs [10], DCs derived from lentivirus-infected individuals may fail to undergo appropriate maturation. Therefore, the low expression level of CD1a may be associated with the
Fig. 3. Uptake of DX-FITC in DCs and macrophages derived from FIV-infected cats and healthy controls. Adherent PBMCs derived from FIV-infected cats (a and c) or healthy controls (b and d) were cultured in the presence (a and b) or absence (c and d) of cytokines for 6 days. These non-stimulated immature DCs and macrophages were incubated with DX-FITC at 0°C (light solid line) or 37°C (thick solid line) for 1 hr. The cells were also incubated at 37°C with mannan (dashed line) to inhibit the mannose receptor.

Fig. 4. Allogeneic T lymphocyte stimulatory capacity of DCs and macrophages derived from FIV-infected cats and healthy controls. Allogeneic PBMCs (5 x 10⁴ cells) derived from healthy controls were stimulated with different numbers of DCs and macrophages that had been treated with mitomycin C. The cells were pulsed with BrdU on day 3, and the proliferation was then estimated. DCs (solid lines and filled symbols) and macrophages (dashed lines and open symbols) were prepared from monocytes derived from FIV-infected cats (a) and healthy controls (b). The results are expressed in terms of absorbance values.
development of immunodeficiency syndrome in FIV and HIV infections. In previous reports [5, 19], it has been shown that CD1a-positive DCs release IL-12 and direct differentiation of T-helper (Th) cells towards the Th1 phenotype, whereas CD1a-negative DCs produce IL-10 and induce differentiation towards the Th0/Th2 phenotype. At present, it remains unknown whether the efficacy of a DC-based immunotherapy is influenced by the low expression level of CD1a. However, DCs derived from FIV-infected cats are expected to possess the potential to act as APCs because they can take up dextran and induce proliferation of allogeneic T cells as shown in the present study.

CD11b, a member of the beta-2 integrin family, combines with CD18 to form the integrin Mac-1 [11]. In the present study, CD11b was expressed on DCs from FIV-infected cats but not on those from healthy controls. This result contradicted previous reports showing that CD11b was detected on feline DCs [4, 27]. The reason for the down-regulation of CD11b on DCs from the healthy controls remains to be solved.

Expression of CXCR4 was detected on DCs derived from both FIV-infected cats and healthy controls. Since CXCR4 is essential for FIV entry into host cells, both DCs and macrophages may be potential target cells for FIV; this observation is in agreement with a previous report that mentions that dendritic-like cells were infected by FIV [23, 31]. However, the p24 protein, which is composed of the FIV Gag protein, was not detected by FCM analysis on the DCs derived from the FIV-infected cats in the present study. Because monocytes were not completely purified from PBMCs in our protocol, contamination of the lymphocytes, which were the main target cells for FIV, rendered PCR analysis unsuitable for detection of viral DNA integration. Further studies are required to confirm whether the DCs were infected by FIV.

In the present study, we confirmed the function of DCs using the endocytosis and MLR assays. DCs derived from FIV-infected cats possessed the ability to take up antigens in a mannose receptor-dependent manner that was similar to that of healthy controls. In the MLR assay, DCs from FIV-infected cats as well as those from healthy controls were shown to activate the proliferation of allogeneic T cells. These results indicate that monocytes derived from FIV-infected cats can differentiate into functional DCs. Similar observations have been reported in cases of HIV infection. In HIV-infected patients, the number of DCs decreases as the disease progresses [9, 14]; however, their antigen presenting functions are not impaired [18]. On the other hand, monocytes derived from HIV-infected as well as non-infected patients can differentiate into DCs in vitro and possess normal properties [6, 26].

In the present study, macrophages could also stimulate proliferation of allogeneic T cells, although they are not considered to be potent activators of allogeneic T cells [4]. The present study showed that both DCs and macrophages expressed the same level of CD80 and MHC class II molecules on their surfaces, indicating that both of these cells possess a similar MLR. Further experiments with DCs and macrophages cultured with cytokines are required to assess their capacities to induce CTLs, since macrophages cultured without cytokines could not take up antigens.

In conclusion, the present study shows that DCs can be generated from PBMCs in FIV-infected cats. These DCs can take up antigen and stimulate allogeneic lymphocytes. These results suggest the possibility of a DC-based immunotherapy against FIV infection. Further work is required to enable induction of an antigen-specific CTL response by pulsing these DCs with FIV antigen.

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