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Low Stimulatory Capacity of Lymphoid Dendritic Cells Expressing Hepatitis C Virus Genes

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To determine the role of antigen presenting cells (APCs) during hepatitis C virus (HCV) infection, murine lymphoid dendritic cells (LDCs), the most potent APCs, were transfected with HCV genes using adenovirus vector-mediated gene transfer. At a multiplicity of infection (MOI) of 100, more than 90% of the transfected LDCs expressed the HCV genes (encoding core-E2 region). The stimulatory capacity of these LDCs (LDC-AxCA327) in the allogeneic mixed leukocyte reaction (Allo MLR) was significantly lower ($P < 0.01$) than that of LDCs infected with control adenovirus vector lacking the HCV genes (LDC-Axw1). LDC-AxCA327 also produced significantly lower levels of IL-12 than LDC-Axw1 ($P < 0.05$). These findings suggest that the inadequate APC capability of LDCs that express HCV genes is related to immunopathology during HCV infection and that IL-12 appears to be involved in this process.

Key Words: **lymphoid dendritic cell; hepatitis C virus; adenovirus vector; interleukin-12; APC; allogeneic mixed leukocyte reaction.**

The hepatitis C virus (HCV) is one of the two most important viruses responsible for persistent infection in humans, and causes chronic hepatitis, cirrhosis of the liver and hepatocellular carcinoma (1, 2). The mechanism underlying the progression of liver injury due to HCV infection remains to be elucidated, however recent studies of the immunopathology of HCV infection suggest that hepatocellular destruction due to HCV is mediated by HCV-specific cytotoxic T cells (3,4,5). These HCV-specific cytotoxic T cells are the final product of a complex set of interactions between T cells, HCV-related antigen and antigen presenting cells (APCs) (6). Lymphoid dendritic cells (LDCs) are the most potent APCs for the primary immune response and are also vital for the development of cytotoxic T cells (7, 8). Depletion, dysfunction and destruction of LDCs has been reported in various infectious diseases, including human HIV infection (9–11). In addition, the inability of the host to eradicate viruses has been attributed to defective LDCs and this might influence the development of persistent viral infection. Peripheral blood mononuclear cells have been found to be infected during HCV infection (12, 13). However, whether HCV gene products affect the APC function of dendritic cells remains to be determined.

The functional characterization of LDCs in HCV-carriers could not be performed until recently due to some obvious technical limitations. On the one hand, there are formidable technical limitations to the isolation of pure populations of fresh LDCs from human HCV-carriers, and on the other hand, there is no suitable animal model of the HCV-carrier state. LDCs have been well characterized in the murine system, and a specific marker of murine LDC has been discovered (14). However, HCV has only been able to infect humans and chimpanzees (15).

To determine the impact of expression of HCV gene products on the function of LDCs, pure populations of LDCs were transfected with HCV genes using an adenovirus (Ad) vector. The successful expression of the HCV genes in these LDCs led us to examine the APC function of LDCs expressing HCV genes.

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Abbreviations used: HCV, hepatitis C virus; LDC, lymphoid dendritic cell; MOI, multiplicity of Infection; APC, antigen presenting cell; Ad, adenovirus; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; Allo MLR, allogeneic mixed leukocyte reaction; IL, interleukin; PI, propidium iodide; S.I., stimulation index.

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MATERIALS AND METHODS

Animals. LDCs and lymphocytes were isolated from 6 to 8 week-old C57BL/6 mice (H-2^b) and C3H/He mice (H-2^k) (Charles River Inc., Yokohama, Japan).

Adenovirus vectors. The recombinant Ad vector was generated using the COS-TPC method (16) and propagated into 293 cells, as described (17). Axw1 (Fig. 1, bottom), which was used as the control Ad vector in this experiment, was derived from replication-deficient Ad type 5 and lacked the E1A, E1B and E3 regions. AxCALacZ (Fig. 1, top) (18) and AxCA327 (Fig. 1, middle) were prepared by inserting the structural genes of LacZ (β-galactosidase construct) and HCV genes (encoding from core to E2 region, amino acids 1-810 (19)), respectively, into the Ad E1-deleted region under the control of the CAG promoter (20). Each Ad vector was then added to the lymphocytes at a multiplicity of infection (MOI) of 50 or 100 and incubated for 3 h at 37°C. The cells were washed 3 times with RPMI 1640 containing 10% FCS before use in the functional assays.

Cell preparation. Various cell populations were prepared from murine spleen according to previously described methods (21) with some modifications. Briefly, a collagenase (type III, Worthington Biochemical, Freehold, NJ, USA)-digested single cell suspension of spleen was centrifuged on a dense BPA column (ρ = 1.082) and the cells at the interface collected and adhered to a plastic surface at 37°C for 1.5 h. After continued overnight culture, non adherent cells were collected and depleted of Fc receptor-bearing cells by rosetting with antibody-coated sheep erythrocytes. The Fc-negative populations consisted primarily of LDCs and about 90% of the dendritic cell population was positive for CD11c, a LDC-specific marker (14). Macrophages were collected from Fc-receptor positive cells in the pellet by killing RBCs with 0.83% NH_4Cl solution. Approximately 60% of the cells in the pellet were macrophages by phagocytosis of 0.75 μm fluorescein isothiocyanate (FITC)-labeled latex particles (Polysciences, Warrington, PA). Lymphocytes consisting of T and B (T/B) cells were obtained by passing spleen cells through a Sephadex G-10 column (Pharmacia, Uppsala, Sweden). T cells were isolated from the T/B cells using a T-cell recovery kit (Biotex Lab. Inc., Alberta, Canada). B cells were isolated from the T/B cells by negative selection using Anti-Thy 1.2 antibody (Cedarlane Lab Ltd., Hornby, Ontario, Canada) and low-toxic complement (Cedarlane).

X-gal staining and immunostaining. The cells infected with the AxCALacZ Ad vector were cultured for 24 or 48 h. They were then plated on a silane-coated slide glass at room temperature under humid conditions for 30 min, fixed with 0.25% glutaraldehyde and stained with 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (16). The efficiency of gene transfection into the cells was estimated by counting cells showing positive (blue) staining.

LDCs infected with AxCA327 or Axw1 (Ad vectors) for 3 h were washed thoroughly, and cultured for a further 48 h to allow for expression of the HCV gene products. The cells were then plated on a silane-coated slide glass and fixed in a 1:1 solution of acetone:methanol at -20°C for 10 min. Next, the cells were incubated with rabbit anti-HCV core polyclonal antibody for 2 h at room temperature under humid conditions. After washing with phosphate-buffered saline (PBS), the cells were incubated with FITC-conjugated goat anti-rabbit IgG (Cappel, Durham, USA) for 1 h at room temperature.

Western blotting. The LDCs were infected with AxCA327 (LDC-AxCA327) or Axw1 (LDC-Axw1), and after washing with medium, cultured for 48 h. These LDCs were centrifuged and the pellets suspended in RIPA buffer (150 mM NaCl, 0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 1% sodium dodecyl sulfate (SDS)), and incubated at 100°C for 5 min. After cooling to 4°C, the lysate was centrifuged at 10,000*g* for 10 min. The supernatant was separated via 15% SDS-polyacrylamide gel electrophoresis and transferred onto Immunobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were incubated with biotinylated 5E3 (an anti-HCV core monoclonal antibody (22, 23)), followed by horseradish peroxidase-conjugated streptoavidin (Vector Laboratories, Burlingame, CA, USA) and proteins visualized using the ECL system (Amersham International plc., Buckinghamshire, UK).

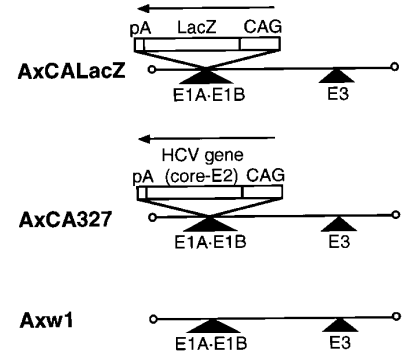

FIG. 1. Structure of adenovirus (Ad) vectors. Solid lines indicate the recombinant Ad genome. A filled triangle below the recombinant Ad genome represents a deletion of Ad regions. Arrows show the orientation of transcription. pA, poly(A) site.

Allogeneic mixed leukocyte reaction (Allo MLR). LDCs (1-5 $\times$ 10^5 cells/ml) were isolated from C57BL/6 mice and infected with AxCA327 or Axw1 for 3 h, or left untreated (LDC-untreated). These LDCs were used as stimulators in the Allo MLR. All LDC populations were treated with mitomycin C (Sigma, St. Louis, MO, USA) for 1 h. Responders consisted of allogeneic T cells (3×10^6 cells/ml) isolated from C3H/He mice. Each 100 μl cell suspension was mixed and cultured in 96-well flat bottomed plates for 4 days. DNA synthesis was assayed by [^{3}H]thymidine (Amersham) incorporation (1 μCi/ml) for the final 14 h of the culture period of 4 days.

Production of interleukin-12 in Allo MLR and by LDCs. The culture supernatants from the Allo MLRs (1×10^6 cells/ml of LDCs and 3×10^6 cells/ml of T cells) were collected after 4 days to assay for interleukin-12 (IL-12). In addition, pure populations of LDCs were cultured for 48 h, and the supernatants collected. The levels of IL-12 from these supernatants were estimated using a commercial ELISA kit (BioSource International, Camarillo, CA).

Cytofluorometry. LDCs infected with an Ad vector were incubated for 48 h after 3 h infection, and directly stained with an optimum dilution of FITC-conjugated specific antibody (purchased from PharMingen, San Diego, CA) or stained with an optimum dilution of primary antibody followed by FITC-conjugated antibody. The cells were then stained with 2 μg/ml propidium iodide (PI) (Sigma). Finally, FITC+ and PI- cells were counted in a flow cytometer (EPICS profile, Coulter Corp., Hialeah, FL). Subclass matched FITC-conjugated mouse IgG and FITC-conjugated secondary antibody alone served as controls.

Statistical analysis. The Student's t-test was used to determine significance differences in mean values between two groups. Significance was established at the $P < 0.05$ level.

RESULTS

Efficient adenovirus-mediated gene expression in APCs. Pure populations of LDCs, macrophages, T cells and B cells were infected with AxCALacZ (Fig. 1, top) at an MOI of 50 and incubated for 48 h. The effi-

TABLE 1

Ratio of β-Galactosidase-Positive Cells That Had Been Transfected with the LacZ Gene via AxCALacZ

Cell type	Ratio of β-galactosidase-positive cells (%) Incubation time after AxCALacZ infection	
	24 hr	48 hr
T cell	1.9 ± 1.3	3.1 ± 3.0
B cell	1.8 ± 1.4	2.0 ± 0.6
Dendritic cell	38.5 ± 8.0	37.8 ± 6.9
Macrophage	33.9 ± 14.4	49.5 ± 7.6

Note. Ratio of positive cells represents the average value of five independent assays ± standard deviation.

ciency of gene transfer was then determined by staining for β-galactosidase, and the expression ratio of the LacZ gene calculated (Table 1). At an MOI of 50, the frequency of expression of the LacZ gene was less than 2 % in T cells and B cells after 24 h incubation, and remained almost unchanged, at 2-3%, after 48 h incubation. In contrast, 30-50% of LDCs and macrophages expressed β-galactosidase at an MOI of 50. When the MOI was increased to 100, expression of the LacZ gene was observed in >90% of LDCs after 48 h incubation (Figs. 2A and 2B).

HCV gene expression in LDCs. The LDCs infected with AxCA327 (Fig. 1, middle), the Ad vector containing HCV genes (encoding core to E2 region) or Axw1 (Fig. 1, bottom) were incubated for 48 h. Expression of HCV-core antigens was confirmed by immunostaining using anti HCV-core polyclonal antibody (Figs. 3A and 3B). Positive HCV-core protein staining was clearly visible in most of the LDC-AxCA327 compared with the LDC-Axw1.

Western blot analysis was performed to determine whether HCV protein production was being carried out by LDCs infected with AxCA327. HCV-core protein (21 kDa) was detected in the LDC-AxCA327 (Fig. 3C), but not the LDC-Axw1.

Low stimulatory capacity of LDC-AxCA327 in Allo MLR. LDCs from C57BL/6 mice, infected with AxCA327 or Axw1 were used as stimulators in the Allo MLR, in which responding T cells were obtained from C3H/He mice (Fig. 4). The results are expressed as the stimulation index (S.I.), which was calculated by dividing the incorporation of [^{3}H]thymidine (cpm) in the presence of LDCs by the incorporation of [^{3}H]thymidine

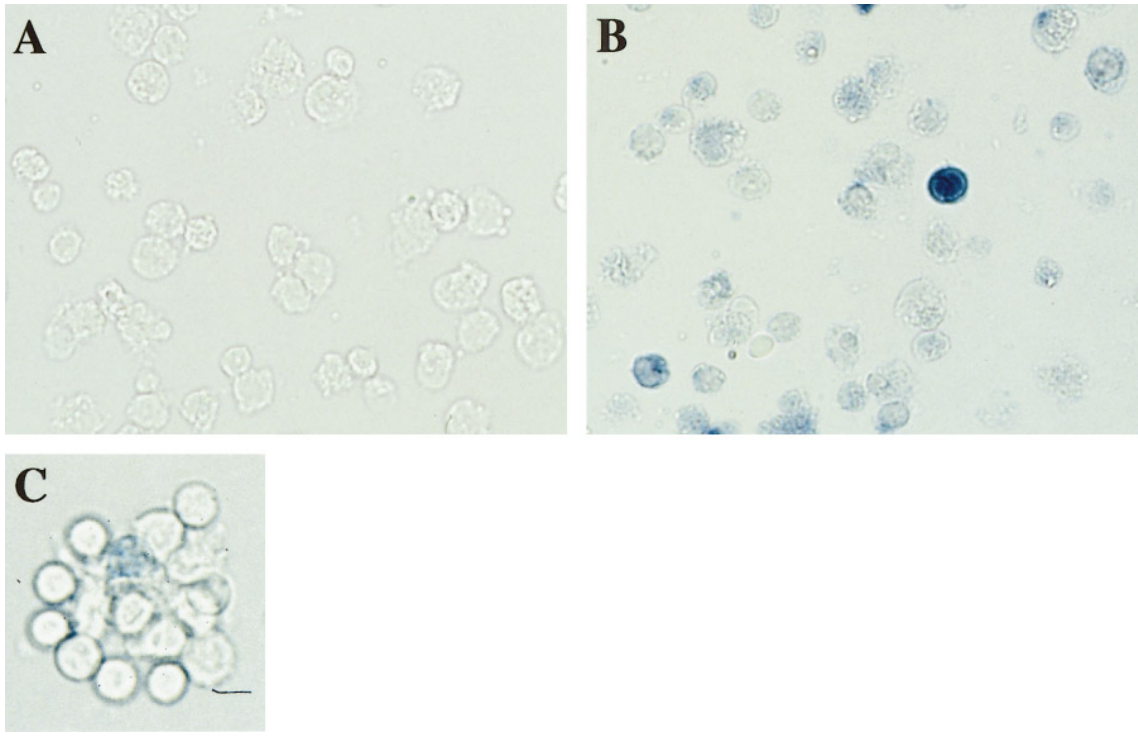

FIG. 2. X-gal staining of lymphoid dendritic cells (LDCs) transfected with AxCALacZ revealed positive expression of β-galactosidase (blue staining) in the LDCs. LDCs were transfected with Axw1 (control) at an MOI of 100 and incubated for 48 h (A), and with AxCALacZ at an MOI of 100 for 48 h (B) (original magnification ×400). (C) X-gal staining of T cells containing LDCs infected with AxCALacZ cultured for 4 days in the allogeneic mixed leukocyte reaction (Allo MLR). LDCs (center of cluster) stained blue, whereas T cells surrounding the LDCs were not stained (×400).

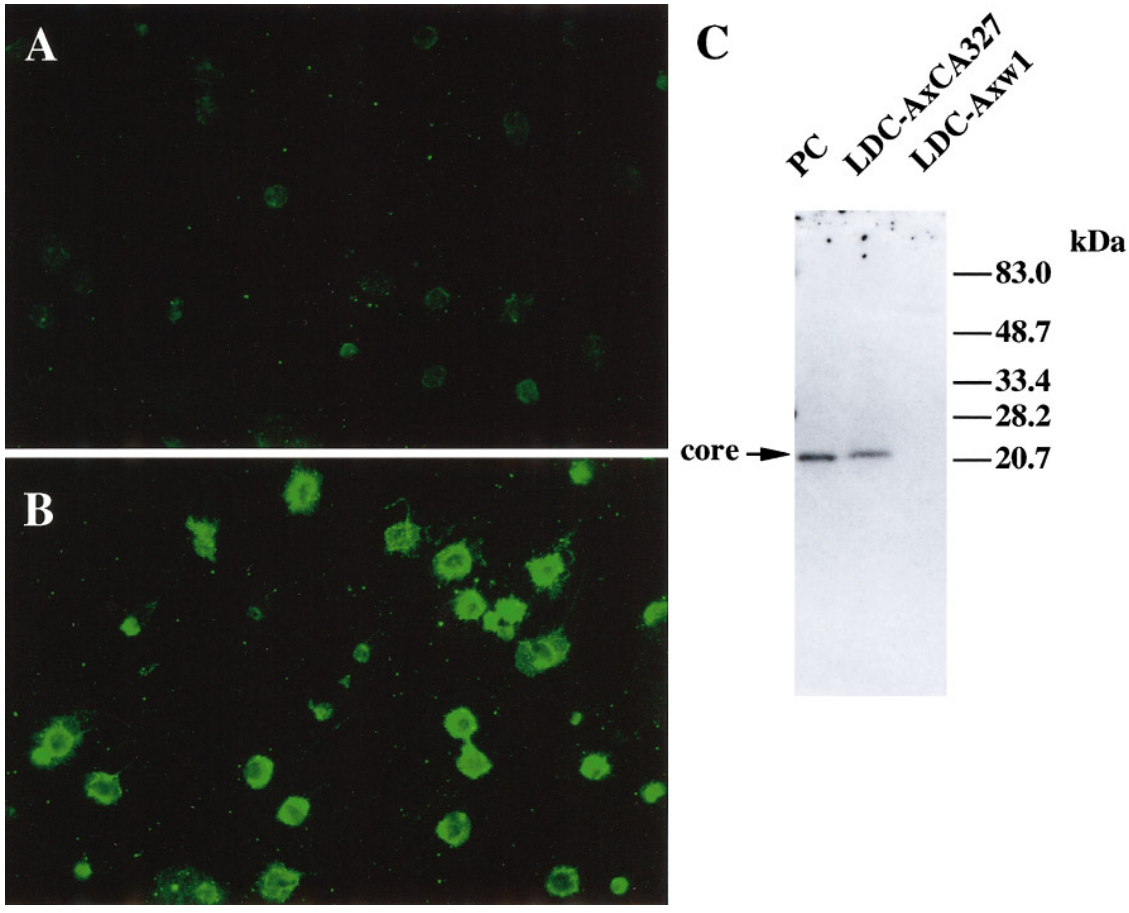

FIG. 3. Immunofluorescence analysis of HCV-core antigens in LDCs transfected with Axw1 (control) at an MOI of 100 and incubated for 48 h (A), and with AxCA327 at an MOI of 100 for 48 h (B) (×400). (C) Western blot analysis of LDCs transfected with AxCA327 (LDC-AxCA327) or Axw1 (LDC-Axw1). PC, positive control core protein expressed in HepG2 cells by full length R6HCV cDNA transfection.

(cpm) by T cells alone (control well). Symbols and bars in Fig. 4 represent the mean ± SE of 9 individual trials. The S.I. of cultures containing LDC-AxCA327 was significantly lower at all doses than cultures containing LDC-Axw1 ($P < 0.01$). There was no significant difference in stimulatory capacity between LDC-Axw1 and untreated LDCs (LDC-untreated) at any dose. The expression of HCV gene products did not influence the viability of cells, as there was no difference in the viability of LDC-AxCA327, LDC-Axw1 or untreated LDCs.

Reduced production of IL-12 by LDCs infected with HCV genes. Interleukin-12 levels were significantly lower in Allo MLR supernatant containing LDC-AxCA327 compared with that containing LDC-Axw1 ($P < 0.05$, $n = 5$) (Fig. 5A). The IL-12 levels were also significantly lower in the supernatants of pure cultures of LDC-AxCA327 compared with that of LDC-Axw1 ($P < 0.05$, $n = 6$) (Fig. 5B).

Expression of Surface Antigens on LDCs. The expression of MHC class II (I-A), H-2K, H-2D, B7-1, B7-2, and ICAM-1 on LDCs infected with Ad vector were determined by cytofluorometry after a 48 h incubation (Fig. 6). The expression of these surface molecules, as shown by mean fluorescence intensity, did not differ significantly between LDC-AxCA327, LDC-Axw1, and untreated LDCs.

DISCUSSION

The Ad vector transfer system is one of the most effective methods of HCV gene transfection. However, the efficiency of gene transfer depends greatly on the cell type being transfected. The rate of gene transfection to T cell lines has usually been poor (17). In the present study, the efficiency of transfection was significantly higher in LDCs and macrophages compared with T and B cells (Table 1, Figs. 2A and 2B). As shown by infection with AxCALacZ, only 2-3% of lymphocytes expressed the LacZ gene at an MOI of 50, whereas about 40% of the LDCs and macrophages expressed β-

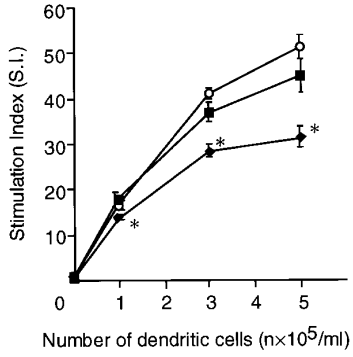

FIG. 4. Proliferation of T cells in the Allo MLR with LDCs as stimulators. Purified T cells (3×10^6 cells/ml) from C3H/He mice were cultured with graded doses (1, 3, 5×10^5 cells/ ml) of untreated LDCs (○), LDCs infected with Axw1 (■), or LDCs infected with AxCA327 (♦) from C57BL/6 mice. Symbols and bars represent mean ± SE (n = 9). *Stimulation index (S.I.) from the Allo MLR is significantly different from Allo MLRs containing LDCs infected with Axw1 or LDCs infected with AxCA327 ($P < 0.01$).

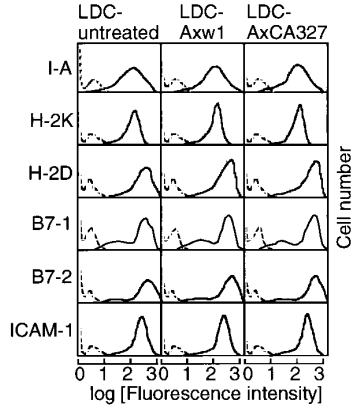

FIG. 6. Cytofluorometric analysis of LDCs transfected with AxCA327 or Axw1, or untreated LDCs. Solid line indicates LDCs stained by fluorescein isothiocyanate (FITC)-conjugated antibody, and dotted line indicates controls stained with subclass matched FITC-conjugated IgG or FITC-conjugated secondary antibody alone.

galactosidase under the same conditions. Moreover, at an MOI of 100, >90% of the LDCs expressed β-galactosidase. This efficient transfection of LDCs using an Ad vector might be due to the unique morphological features of LDCs (dendritic processes have the capacity to form clusters), or due to LDCs being rich in adhesion molecules and integrins (6). However, the underlying mechanism remains to be clarified.

The stimulatory capacity of LDC-AxCA327 was significantly lower than that of LDC-Axw1 in the Allo MLR at all LDC doses, whereas the stimulatory capacity of LDC-Axw1 and untreated LDCs did not differ significantly at any dose (Fig. 4). This decrease in stimulatory capacity of LDC-AxCA327 was not due to a change in viability resulting from transfection. In the Allo MLR, the formation of clusters between T cells and LDC is the vital step because non clustered cells show a very low degree of proliferation. Thus, during T cells/LDC clustering, the Ad vector may have entered the T cells and blocked proliferation. However, this possibility was ruled out by X-gal staining of T cells containing LDCs infected with AxCALacZ collected 4 days after the start of Allo MLR, which showed no staining of T cells due to infection with AxCALacZ in the Allo MLR (Fig. 2C). Another possibility is that AxCA327 may be more cytotoxic than Axw1. This was examined using another Ad vector (AxCANcre, an Ad vector which expresses Cre DNA recombinase (15)). The results did not support the notion of increased AxCA327 cytotoxicity (data not shown). Thus, HCV antigen was found to be the primary factor responsible for the reduced stimulatory capacity of LDC-AxCA327 in the Allo MLR.

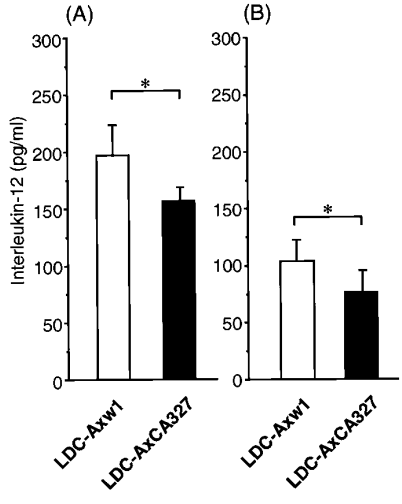

FIG. 5. Low levels of interleukin-12 (IL-12) production by LDCs, transfected with AxCA327. LDCs were transfected with AxCA327 and Axw1, and after infection were cultured for 4 days to allow for IL-12 production in the Allo MLR (A) and for 48 hr in pure cultures (B). LDCs transfected with AxCA327 (■) produced significantly lower levels of IL-12 compared with LDCs, transfected with Axw1 (□). Bars indicated Mean ± SD. *$P < 0.05$.

The low stimulatory capacity of LDC-AxCA327 in the Allo MLR was analyzed by examining their capacity to produce IL-12, because IL-12 is known to induce T cell proliferation (24, 25) and is the only vital cytokine produced by LDCs. IL-12 levels in the supernatant of the Allo MLR containing LDC-AxCA327 were significantly lower than in the Allo MLR containing LDC-Axw1 (Fig. 5A). Although the IL-12 in this Allo MLR was most likely produced entirely by the LDCs, we decided to confirm this by culturing LDCs for 48 h and measuring the spontaneous production of IL-12 in culture by pure populations of LDCs. LDC-AxCA327 produced signifi-

cantly lower amounts of IL-12 compared with LDC-Axw1 (Fig. 5B).

In general, Ad is a highly replicative virus, and expresses gp19K protein which can inhibit the immune response by combining with cytoplasmic MHC class I (26). However, the Ad vectors in the present study were replication defective due to the lack of E1 (Fig. 1). Moreover, these vectors lack the E3 region, which contributes to the production of gp19K. In addition, the viability of cells transfected with Ad vectors was not affected by the transfection.

We report the successful transfection of LDCs with HCV genes, and expression of HCV antigens in LDCs. The preferential expression of HCV antigens in LDCs compared with lymphocytes, and the low stimulatory capacity of LDC-AxCA327 in the Allo MLR suggest a specific defect in immune regulation upon HCV infection, which may be associated with the pathogenesis of persistent HCV infection. To determine the clinical implications of this observation, experiments in vivo using HCV transgenic mice are underway. A better understanding of the mechanism of immune dysfunction in this system would contribute to the development of new strategies to fight HCV infection.

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