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Interaction of Rotavirus with Human Myeloid Dendritic Cells

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We have previously shown that very few rotavirus (RV)-specific T cells that secrete gamma interferon circulate in recently infected and seropositive adults and children. Here, we have studied the interaction of RV with myeloid immature (IDC) and mature dendritic cells (MDC) in vitro. RV did not induce cell death of IDC or MDC and induced maturation of between 12 and 48% of IDC. Nonetheless, RV did not inhibit the maturation of IDC or change the expression of maturation markers on MDC. After treatment with RV, few IDC expressed the nonstructural viral protein NSP4. In contrast, a discrete productive viral infection was shown in MDC of a subset of volunteers, and between 3 and 46% of these cells expressed NSP4. RV-treated IDC secreted interleukin 6 (IL-6) (but not IL-1β, IL-8, IL-10, IL-12, tumor necrosis factor alpha, or transforming growth factor beta), and MDC released IL-6 and small amounts of IL-10 and IL-12p70. The patterns of cytokines secreted by T cells stimulated by staphylococcal enterotoxin B presented by MDC infected with RV or uninfected were comparable. The frequencies and patterns of cytokines secreted by memory RV-specific T cells evidenced after stimulation of peripheral blood mononuclear cells (PBMC) with RV were similar to those evidenced after stimulation of PBMC with RV-infected MDC. Finally, IDC treated with RV strongly stimulated naive allogeneic $CD4^+$ T cells to secrete Th1 cytokines. Thus, although RV does not seem to be a strong maturing stimulus for DC, it promotes their capacity to prime Th1 cells.

Rotaviruses (RV) are responsible for the deaths of approximately 1,200 children daily worldwide (41). To develop new strategies for treating or preventing this disease, our laboratory is currently characterizing human virus-specific T and B cells in children and adults (16, 23, 24, 48). We recently showed that healthy adults have very low frequencies of circulating $CD4^+$ and $CD8^+$ RV-specific T cells that secrete gamma interferon (IFN-γ) (23, 48). Significantly higher frequencies of these cells were found in symptomatic RV-infected adults. Nonetheless, the frequencies of these cells in all groups of study volunteers were relatively low compared to the frequencies of T cells specific for other pathogens (23, 48). Surprisingly, in RV-infected children, virus-specific $CD4^+$ and $CD8^+$ T cells that secreted IFN-γ, interleukin 13 (IL-13), or IL-4 were very low or undetectable. From these studies, we hypothesized that RV has developed mechanisms to evade the immune response (23). In agreement with this hypothesis, sterilizing immunity is not induced after primary RV infection (11, 53). However, children develop a lymphoproliferative response to RV after primary infection (36, 40), and over 90% of adults have a response of IFN-γ-secreting T cells in response to RV (27). These results suggest that if RV has developed immune evasion mechanisms, they are only partial in RV-infected children and can eventually be overcome after multiple reinfections in adults.

Viruses have evolved strategies to evade the immune system by altering the function of dendritic cells (DC), which are key players in antiviral innate immunity and the development of acquired immune responses (31). Localized in the peripheral organs in an immature state (IDC), they serve as sentinels of the immune system waiting for the entrance of pathogens (30). When an IDC encounters a pathogen it "matures" and migrates to secondary lymphoid organs, where it presents processed antigens to specific T cells. This process of DC differentiation has been partially simulated in vitro (3). Cells with the phenotype of the interstitial IDC ($CD14^-$ $CD83^-$ HLA-DR^+ $CD86^{+/-}$) localized in the peripheral tissues are generated from circulating monocytes ($CD14^+$) cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (3). These cells have a high capacity to endocytose antigens but are relatively inefficient as antigen-presenting cells (APC) (30). When these IDC are infected in vitro with pathogens (7, 18, 35) or stimulated with proinflammatory cytokines (tumor necrosis factor alpha [TNF-α] or IL-1) (46), prostaglandin E_2 (PGE_2) (46), products of bacterial (lipopolysaccharide [LPS]) (47) or viral (double-stranded RNA [dsRNA]) (7) origin, or antibodies against CD40 (7), they acquire the phenotype of mature dendritic cells (MDC) ($CD14^-$ $CD83^+$ HLA-DR^{++} $CD86^{++}$). MDC lose their capacity to endocytose antigens and become the most efficient APC known. Viruses evade the immune response by neutralizing the function of DC through many different mechanisms (reviewed in reference 31), for example, (i) induction of apoptosis of DC (1, 7, 10), (ii) inhibition of the maturation of IDC (10, 17, 49), (iii) direct inhibition of the antigen presentation pathway of DC (44), (iv) induction of production of cytokines by DC that inhibit the development of virus-specific T cells (17), and (v) induction of the expression of molecules on DC (FasL and TRAIL) that cause apoptosis in T cells (51).

The APC involved in inducing the T-cell responses to RV are poorly understood. We have previously shown that human monocytes treated in vitro with RV are able to present antigen to specific $CD4^+$ T cells (48). Recently, it was also reported that DC treated with RV can stimulate RV-specific T cells from rhesus macaques (52). Our knowledge of the APC that

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probably present RV antigen in vivo comes from animal models: viral antigen has been detected in the Peyer's patches, mesenteric lymph nodes, and spleens of mice up to 20 days after RV infection (8). In these organs, viral antigen was mostly localized to the cytoplasm of DC (identified by the expression of major histocompatibility complex class II molecules, and absence of Mac-1 and surface immunoglobulin), but also macrophages and B cells, suggesting that these cells have the capacity to present viral antigen to RV-specific T cells. A similar study was able to demonstrate viral antigen in macrophages and B cells but not in $CD11c^+$ (a DC marker) cells (6). Neither of the two studies was able to determine if RV was replicating in the cells where viral antigen was identified.

Here, we have studied the interaction of RV with human myeloid IDC and MDC. We investigated the capacity of RV to induce cell death, modulate the phenotype, infect, and induce the secretion of cytokines by these APC. Finally, we tested the capacity of DC treated with RV to stimulate syngeneic memory and allogeneic naive T cells.

MATERIALS AND METHODS

Isolation of monocytes and generation of IDC and MDC. Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteers who signed informed consent forms approved by the Ethics Committee of the Medical School of the Pontificia Universidad Javeriana. PBMC were isolated by density gradient using Lymphosep medium (ICN Biomedicals, Inc., Irvine, CA). The cells were washed twice with RPMI 1640 (Gibco-BRL, Gaithersburg, MD) supplemented with L-glutamine, 2 mM; HEPES, 20 mM; penicillin, 100 U/ml; streptomycin, 100 mg/ml (Gibco-BRL); and autologous plasma, 1% (complete medium) and resuspended in phosphate-buffered saline (PBS) containing 2 mM EDTA (Sigma Aldrich, St. Louis, MO) plus 0.5% human albumin (Biotest Pharma, Dreieich, Germany). Immunomagnetic negative selection of $CD14^+$ cells was performed using a Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA) following the manufacturer's instructions. Purified $CD14^+$ cells (median purity, 91.3%; range, 82 to 95%; $n = 16$) were used to generate IDC and MDC. For the generation of IDC, 3 million $CD14^+$ cells were cultured at 37°C with 5% CO_2 in 3 ml of complete medium plus 1,000 U/ml GM-CSF (R&D Systems, Minneapolis, MN) and 1,000 U/ml IL-4 (R&D Systems) (3, 45). Three to 5 days later, the cells were washed with PBS-0.5% bovine serum albumin (Sigma)-0.02% sodium azide (Mallinckrodt Chemicals, Paris, KY) (staining buffer) and stained using different combinations of fluorochrome-labeled monoclonal antibodies (MAbs) against the following human proteins: CD14-fluorescein isothiocyanate (FITC) (clone M5E2), CD83-phycoerytherin (PE) (clone HB15e), HLA-DR–peridinin chlorophyl protein (PerCP) (clone L243), CD86-allophycocyanin (clone FUN-1), and isotype control MAbs (BD Bioscience, San Jose, CA). After being stained, the cells were washed once with staining buffer and fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Washington, PA). At least 20,000 cells were acquired on a FACSCalibur (BD Bioscience) equipped with a second 635-nm red diode laser and analyzed with Cellquest software version 3.1. Dead cells and debris were excluded by forward and side scatter gating.

To produce MDC, nonadherent IDC were washed out of cytokine-containing culture media, counted, and incubated in a new 24-well tissue culture plate (Falcon BD Labware) in complete medium with GM-CSF, 1,000 U/ml, and IL-4, 1,000 U/ml, and supplemented with poly($I \cdot C$) (20 μg/ml; Invitrogen, San Diego, CA) for 48 h (7). Poly($I \cdot C$) was chosen as the maturing stimulus because of its resemblance to the RV genome and its known capacity to induce MDC with a good capacity to activate T cells (7). In some experiments, IDC were matured with PGE_2 (10 μM; Sigma) and TNF-α (5,000 U/ml; R&D Systems) for 24 h (46). The phenotype of MDC was confirmed by flow cytometry using the same panel of antibodies described above. Due to the fact that the results of the experiments described above can vary importantly because of the presence of low concentrations of LPS in the cultures (47), all reagents and RV used were analyzed and determined to be free of LPS with a commercial kit (BioWhittaker, Inc., Walkersville, MD). Moreover, the lack of contamination of cultures with LPS was supported by the absence of spontaneous production of TNF-α in cultures of IDC (47).

Production and titration of RRV and Wa RV. The tissue culture-adapted rhesus rotavirus (RRV) was grown in MA104 cells in the presence of trypsin and, in most experiments, used after cesium purification (14). The purified stock virus preparation had a protein concentration of 0.8 mg/ml and a titer of 2×10^{10} focus-forming units /ml. The virus was dialyzed against RPMI before use. A fraction of this dialyzing medium was saved to be used as a negative control (control). Wa RV (human RV) was similarly grown in MA104 and used as a clarified tissue culture supernatant. When unpurified RV were tested, the supernatant of mock-infected MA104 was used as a negative control. The RNA patterns of both RV strains were checked by polyacrylamide gel electrophoresis. MA104 cells were shown to be mycoplasma free with the VenorGeM kit (Sigma).

Evaluation of viability and presence or absence of apoptosis in IDC and MDC treated with RV. IDC and MDC were washed out of cytokine-containing culture media and incubated with cesium-purified RRV using different multiplicities of infection (MOI) or with control for 45 min at 37°C with 5% CO_2. After that, the cells were washed twice and incubated with complete medium without autologous plasma (medium without plasma) with GM-CSF, 1,000 U/ml, and IL-4, 1,000 U/ml, at 37°C with 5% CO_2. Forty-eight hours later, the cells were washed and counted and their viability was evaluated using trypan blue. To establish if RV induces apoptosis and/or necrosis, cells were stained with propidium iodide (Sigma; 0.2 μg/ml) and annexin V (using a commercial kit produced by Molecular Probes Inc., Eugene, OR) for 15 min at room temperature (RT) protected from light and then analyzed by flow cytometry within 60 min (7).

Phenotype of DC treated with RV. IDC or MDC were washed with complete medium and incubated with cesium-purified RRV or control in complete medium, as described above. At different times after treatment, cells were washed, and their phenotype was established by flow cytometry as described above. Results were reported as the mean fluorescence intensity of staining with each marker and the percentage of cells that expressed each or combinations of the different markers.

RV infection of DC. DC were washed, resuspended in complete medium without plasma, and then treated with RRV or control for 45 min at 37°C with 5% CO_2, washed twice, and incubated for different periods of time in complete medium without plasma. At the end of this incubation, supernatants and cell pellets were collected, frozen, and treated as described below. In some experiments, cells were washed with staining buffer and fixed with 1% paraformaldehyde (Electron Microscopy Sciences) for 5 min. Then, they were washed and permeabilized with 1× fluorescence-activated cell sorter permeabilizing solution (BD Bioscience) for 10 min at RT. The cells were washed again and blocked with staining buffer with 10% autologous plasma for 10 min at RT and stained with a MAb against a viral nonstructural protein, NSP4 (MAb B4-2 ascites; supplied by H. B. Greenberg, Stanford University) for 30 min at 4°C. After being washed, the cells were blocked with a solution of normal goat serum-normal human plasma in a 9:1 proportion for 10 min at RT. Then, goat anti-mouse allophycocyanin (Molecular Probes, Eugene, OR) was added for 30 min at 4°C. Cells were washed and incubated with MAbs against CD14-FITC, CD83-PE, and HLA-DR-PerCP. Finally, the cells were washed, fixed with 1% paraformaldehyde, and analyzed by flow cytometry as described above. In some experiments, MDC were infected in the presence of the RV-neutralizing MAb anti-VP7 (MAb 159 ascites; supplied by Harry B. Greenberg) or an isotype control MAb (BD Bioscience).

Detection of replication of RV in DC. In order to establish if RV was able to perform a complete replication cycle, supernatants and cell pellets collected in the above-described experiments were frozen and thawed three times. Virus in the samples was titered in MA104 in the presence of trypsin in 1/5 serial dilutions, as was done with the viral stock (14). In the same samples, the concentration of the viral structural protein VP6 was studied by enzyme-linked immunosorbent assay (ELISA) (23, 48). Briefly, Immulon 2 ELISA plates (DYNEX Technologies, Chantilly, VA) were coated with rabbit anti-RV hyperimmune serum or preimmune serum (Dako Cytomation, Glostrup, Denmark) diluted in PBS and incubated overnight at 4°C. After being blocked, 1/2 serially diluted samples were added and incubated. The wells were washed, and MAb anti-VP6 (MAb 1026 ascites; supplied by Evelyn Kholi, Dijon, France) was added and then developed with a biotinylated goat anti-mouse serum (Vector, Burlingame, CA), followed by a peroxidase avidin-biotin complex kit (ABC Kit; Vector) and tetramethyl benzidine substrate (Sigma, St. Louis, MO). The reaction was stopped by the addition of sulfuric acid. The absorbance was read at a wavelength of 450 nm on an ELISA plate reader.

Measurement of cytokines in cell culture supernatants. The cytokines present in the supernatants of DC treated with RV or untreated were frozen and stored at −70°C. The supernatants were then thawed, and cytokines were measured with commercial kits following the manufacturer's instructions. IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α were detected using a BD cytometric bead array

(CBA) human inflammation kit (BD Bioscience). Activated transforming growth factor beta 1 was quantified by ELISA with an R&D kit following the manufacturer's instructions.

Detection of cytokine-secreting CD4$^+$ T cells. Frequencies of RV-specific CD4$^+$ T cells among PBMC were determined as previously described (23, 48). In addition, MDC infected with RV (MOI = 5) for 24 h or uninfected were mixed at a ratio of 1:10 with fresh autologous PBMC for 10 h in the presence of anti-CD28 (0.5 μg/ml) and anti-CD49d (0.5 μg/ml) MAbs (BD Bioscience) as costimulators. The last 5 h of the incubation included brefeldin A (10 μg/ml) (Sigma) to block the secretion of cytokines from the cell. At the end of the incubation, the cells were washed, fixed with 1% paraformaldehyde (Electron Microscopy Sciences), and then frozen. Cells were thawed, incubated with fluorescence-activated cell sorter permeabilization solution (BD Bioscience), and then stained in the dark with the following MAbs: anti-CD4–PerCP, anti-CD69–allophycocyanin, anti-IFN-γ–FITC or anti-IL-2–FITC, and anti-IL-13–PE or anti-IL-10–PE (BD Bioscience). Isotype-matched MAbs labeled with FITC and PE obtained from BD Bioscience were used as controls.

In order to establish if RV infection could modulate the pattern of cytokines secreted by polyclonal T cells, MDC infected with RV or uninfected were treated with the superantigen staphylococcal enterotoxin B (SEB) (Sigma), 1.25 μg/ml, for 30 min at 37°C. The cells were then washed and incubated with fresh autologous PMBC as described above.

Stimulation of allogeneic naive CD4$^+$ T cells. Naive CD4$^+$ CD45RA$^+$ T cells were purified using a negative selection isolation kit (Miltenyi) following the manufacturer's instructions. The purity of the naive cells obtained was greater than 93% (n = 3). IDC (obtained as described above) treated with RV (MOI = 5) for 24 h or untreated were mixed at a ratio of 1:10 with the purified allogeneic CD4$^+$ T cells for 7 days (7). The cells were then washed and stimulated with phorbol myristate acetate (PMA) (50 ng/ml; Sigma) and A23187 Ca^{2+} ionophore (500 ng/ml; ICN Biomedicals) for 4 h in the presence of brefeldin A (10 μg/ml; Sigma). At the end of the incubation, the cells were washed, fixed with 1% paraformaldehyde (Electron Microscopy Sciences), and stained for analysis of intracellular cytokines as described above for memory T cells.

Statistical analysis. Statistical analysis was performed with SPSS software version 10.0 (SPSS Inc., Chicago, IL). Medians and ranges of results for the different experimental groups are reported. Differences between unpaired and paired results were determined with the Mann-Whitney and the Wilcoxon tests, respectively. Significance was established if P was <0.05. For statistical analysis of results quantifying cytokines by CBA (see Fig. 4B), values below the limit of sensitivity of the assay were assigned a value of one-third of the sensitivity limit.

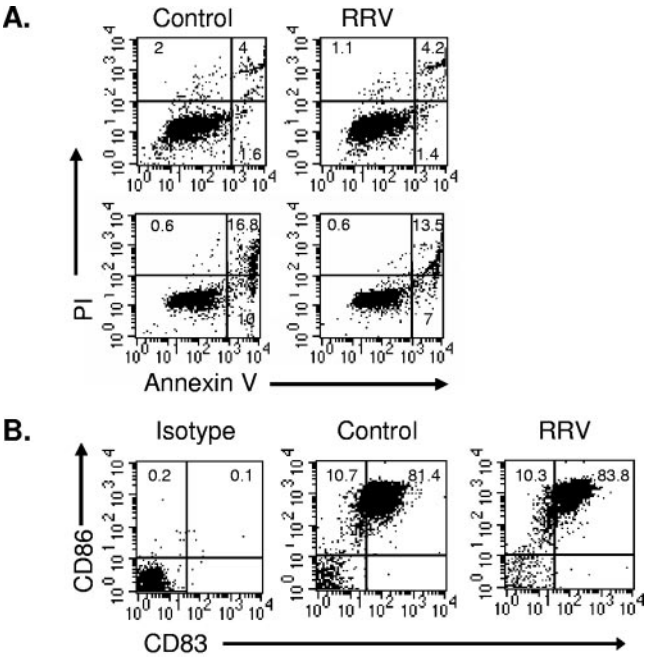

FIG. 1. RV does not induce cell death in IDC and MDC or inhibit the maturation of IDC. (A) Levels of expression of propidium iodide (PI) and annexin V were evaluated in the two subsets of DC 48 h after treatment with the control preparation or RV at an MOI of 5. The percentages of cells in the upper and right quadrants are indicated. The results are representative of five experiments performed. (B) IDC were treated with control or with RV at an MOI of 5 and 24 h later treated with poly(I · C). Levels of expression of CD86 and CD83 were evaluated 24 h later. The percentages of cells in the upper quadrants are indicated. The results shown are representative of two experiments performed.

RESULTS

Rotavirus does not induce cell death or inhibit the maturation of DC. Some viruses evade the immune system by inducing the death of DC (2). The proportions of dead IDC and MDC treated with RRV at an MOI of 5 or 10 or untreated, as evidenced by trypan blue staining, were similar 2, 5, 24, and 48 h after treatment (n = 2 to 14 for each condition) (data not shown). To confirm these results and to determine if treatment of DC with RV induced apoptosis, we stained control or RV-treated (MOI = 5) DC with propidium iodide and annexin V 48 h after treatment. As can be seen in Fig. 1A, frequencies of PI$^+$ (necrotic) and annexin V$^+$ (apoptotic) cells were similar before and after either treatment. No statistical differences were noted between the frequencies of annexin V$^+$ or PI$^+$ APC treated with RV or untreated (n = 5; Wilcoxon test, $P >$ 0.5). Similar results were observed with IDC (n = 1) and MDC (n = 2) at 24 h and 48 h posttreatment with RV with an MOI of 10 (data not shown). Thus, RV does not seem to induce cell death in DC.

To determine if RV can modulate the phenotypes of IDC and MDC, we treated these cells with control or cesium-purified RRV at an MOI of 5 for 24 h and evaluated the expression of CD14, HLA-DR, CD86, and CD83 by flow cytometry. For comparison, we also stained freshly isolated monocytes and subsets of DC before treatment (Table 1). As expected, only freshly isolated monocytes expressed CD14 (Table 1), showing that in our cultures, the initial DC maturation step had occurred. Analysis of the relative quantities of CD83, CD86, and HLA-DR expressed by the different DC, based on the mean fluorescence intensity of staining, permitted us to conclude that RV induces a modest maturation of IDC and does not modulate the phenotype of MDC (Table 1). RV induced a net (the percentage of IDC treated with RV minus the percentage of IDC treated with the control preparation) median of 18.4% (n = 10; range, 12 to 48%) IDC to become MDC that expressed both CD83 and CD86. Treatment of IDC with RRV at an MOI of 10 for 24 h or with an MOI of 5 for 48 h induced a change in phenotype similar to that induced by the treatment with an MOI of 5 for 24 h shown in Table 1 (n = 2 for each condition) (data not shown). We conclude that, while RV induces the maturation of approximately one-fifth of IDC, it does not induce changes in the phenotype of MDC.

The results described above did not rule out the possibility that RV could be inhibiting the maturing effect of exogenous stimuli. To test this hypothesis, we cultured IDC with RRV and then treated them with poly(I · C) 24 h later (n = 2) or simultaneously added the poly(I · C) with RV (n = 1). RV did not inhibit the maturing effect of poly(I · C) under either condition (Fig. 1B and data not shown). The effect does not seem to be specific for poly(I · C), as RV administered simultaneously with PGE_2 and TNF-α also did not inhibit the ca-

TABLE 1. Expression of maturation markers by DC treated with control or with RV

Treatment group	Expression[a]			
	CD14	HLA-DR	CD86	CD83
Monocytes	69 (26–212)	29 (12–44)	16 (2–30)	3 (2–4)
IDC initial	17 (12–23)	240 (95–288)	53 (8–136)	11 (7–37)
IDC + control	19 (12–25)	358 (145–1331)	107 (70–337)	25 (13–99)
IDC + RRV	19 (12–23)	716 (322–1612)*	329 (139–566)*	49 (27–155)*
MDC initial	25 (18–33)	1,593 (802–2051)	1,212 (460–1485)	213 (96–266)
MDC + control	24 (16–32)	1,521 (804–2802)	1,463 (429–3842)	249 (137–457)
MDC + RRV	24 (20–33)	1,470 (944–2974)	1,364 (446–3507)	202 (192–470)

[a] Monocytes and DC prior to treatment (initial) or 24 h after treatment with the control preparation (control) or RV (RRV) were stained with antibodies against CD14, HLA-DR, CD86, and CD83 as in Fig. 2. Values are given as the median (range) of the mean fluorescence intensity of staining for each marker. *, statistically significant difference between RV- and control-treated APC (Wilcoxon test; $P < 0.05$; $n = 10$ experiments for DC and 12 experiments for monocytes).

pacity of this stimulus to induce maturation of DC ($n = 2$) (data not shown). Thus, RV is not a potent agent for maturation of IDC, but it does not inhibit the process.

MDC are productively infected with RV. To determine if IDC and MDC were infected with RV, we treated the cells with RRV at an MOI of 5, and 2 or 24 h later, the cells were permeabilized and stained with a MAb against NSP4 (a nonstructural protein) and CD83. No expression of NSP4 was evident in IDC ($n = 1$) and MDC ($n = 3$) 2 h after treatment with RRV (data not shown). Nonetheless, as can be seen in Fig. 2A and B, 24h after treatment an important fraction of MDC expressed both CD83 and NSP4. In contrast, few IDC expressed NSP4 (Fig. 2A and B). The frequency of NSP4-expressing IDC and MDC did not increase if evaluation of NSP4 was done 48 h after infection ($n = 2$) (data not shown). The frequency of NSP4-expressing cells was also not increased

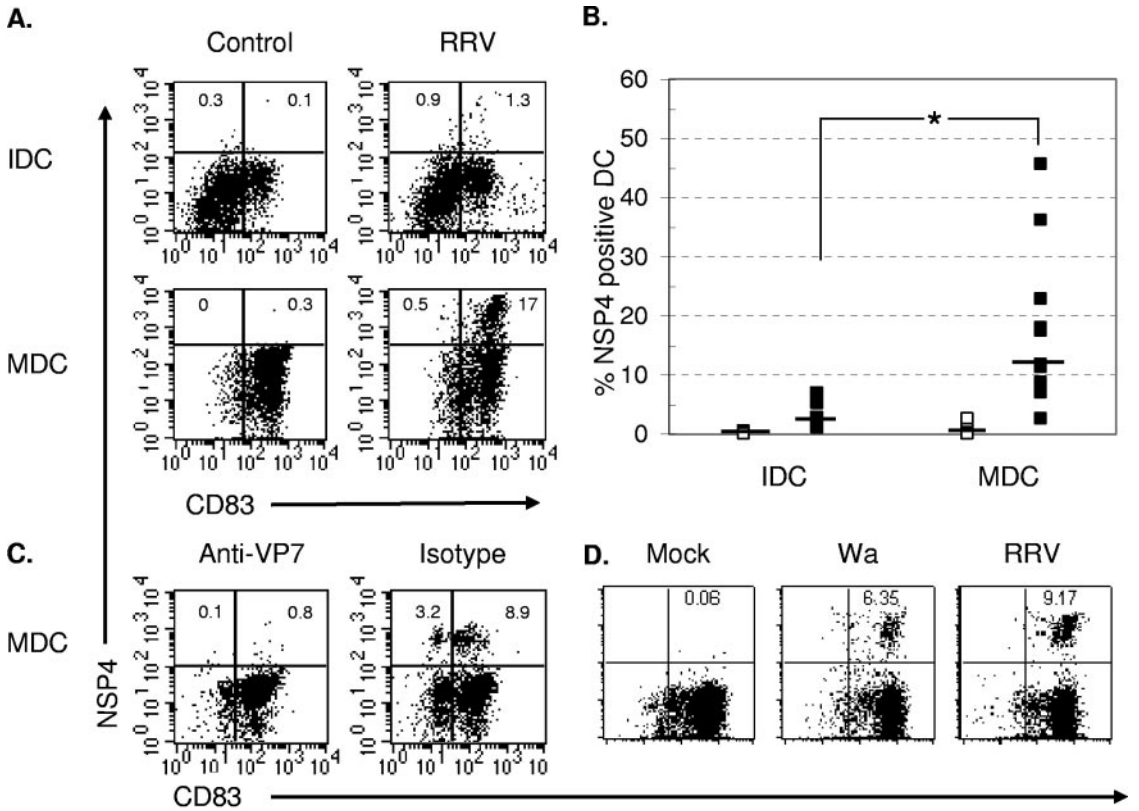

FIG. 2. Expression of NSP4 by IDC and MDC treated with RV. (A) DC were treated with control or purified RRV (MOI = 5) for 24 h. The cells were then permeabilized and stained with MAbs against NSP4 and CD83. (B) Summary of experiments presented in panel A. Open squares, DC treated with control; closed squares, DC treated with RRV. Results from 11 and 12 experiments with IDC and MDC are presented. The lines represent medians. The asterisk indicates a statistical difference between the frequencies of IDC and MDC expressing NSP4 ($P < 0.001$; Mann-Whitney test). (C) MDC were infected with RV (MOI = 5) in the presence of the neutralizing anti-VP7 MAb 159 or of an isotype control MAb. (D) MDC were treated with the supernatant of mock-infected or RRV- or Wa RV-infected MA104 cells at an MOI of 5. The results in panels C and D are representative of three experiments performed. The percentages of cells in the in the upper quadrants of the dot plots are indicated.

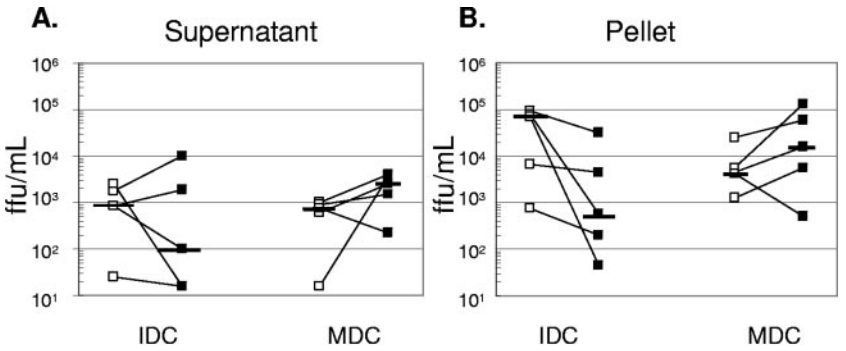

FIG. 3. Productive infection of MDC with RV. The supernatants (A) and cell pellets (B) of IDC and MDC cultures 2 h (open squares) and 48 h (closed squares) after treatment with RV were titered on MA104 cells. The results of five individual experiments are united by a line. The horizontal lines represent medians.

if IDC treated with RV were matured with poly(I · C) 24 h later, suggesting that induction of maturation after infection does not increase viral replication ($n = 2$) (data not shown). DC matured with TNF-α and PGE_2 were also infected with RV at similar levels ($n = 2$) (data not shown), showing that infection did not depend on the maturation stimulus used to obtain the MDC. Simultaneous incubation of MDC with RRV and the 159 (anti-VP7) neutralizing MAb, but not an isotype control MAb, completely blocked infection of MDC (Fig. 2C), suggesting that VP7 is important for viral entry into MDC. Finally, we treated IDC and MDC with tissue culture supernatant of RRV- and Wa human RV-infected cells. Compared to RRV, the human RV infected IDC and MDC to similar or lower levels (Fig. 2D and data not shown), showing that homologous virus can also infect IDC and MDC.

To determine if the infection of DC was productive, we evaluated infectious virus in the supernatants and cell pellets of IDC and MDC at 2 and 48 h after treatment with RRV. With MDC from four out of five volunteers, an increase in viral titers in the supernatants and pellets of cell cultures was seen (Fig. 3). The increase in viral titers varied from between approximately 2-fold to 200-fold, suggesting that the viral replication efficiency is variable and considerably lower than that seen with classical cell lines used to grow RV. In contrast, in similar experiments with IDC, changes in viral titers generally decreased (Fig. 3). In the same cultures, a statistically significant (Wilcoxon test, $P < 0.05$) increase in the relative quantities of VP6 in both the pellet and the supernatant of the MDC cultures was also evident (Table 2). We could not detect similar increases in relative quantities of VP6 in cultures of IDC (Table 2). Thus, low productive RV infection seems limited to MDC of a subset of volunteers, and very little if any viral replication is seen in IDC.

TABLE 2. De novo synthesis of VP6 by MDC, but not IDC treated with RV

Cell type	Relative quantities of VP6 (ELISA OD_{450})[a]			
	Supernatant		Pellet	
	2 h	48 h	2 h	48 h
IDC	47 (0–92)	98 (0–213)	50 (0–70)	73 (0–195)
MDC	19 (10–43)	207 (129–865)*	30 (0–68)	165 (136–546)*

[a] DC were treated with RV for 2 or 48 h. Relative quantities of VP6 were evaluated in the supernatants and cell pellets of IDC and MDC by ELISA. Values are given as median (range); $n = 5$. OD_{450}, optical density at 450 nm.

Cytokines secreted by IDC and MDC treated with RV. We next examined the cytokines produced by DC treated with RRV. We first measured the quantities of cytokines present in the supernatants of cultures used to produce IDC and MDC (Fig. 4A). This pattern of cytokine secretion is similar to that reported previously (39) and demonstrates that in our experiments, DC were capable of producing the cytokines evaluated. We next compared the concentrations of cytokines present in supernatants of DC treated with RRV or untreated (Fig. 4B). RV-treated IDC and MDC secreted significantly larger amounts of IL-6 ($n = 6$; Wilcoxon test, $P < 0.042$). MDC also secreted small but significantly larger amounts of IL-10 and IL-12p70 ($n = 7$; Wilcoxon test, $P = 0.043$). However, the levels of both cytokines produced were relatively low (Fig. 4B). Although not significantly different, IDC treated with RV also tended to produce more IL-10, and the two types of DC were prone to secrete more TNF-α. None of the two types of DC treated with RV secreted significantly larger amounts of IL-1β or IL-8 (Fig. 4B) or activated transforming growth factor beta (data not shown).

Infection of MDC with RV does not modify their capacity to stimulate polyclonal $CD4^+$ T cells. The production of IL-10 and low-level production of IL-12 by MDC infected with RV suggested that these APC would not support strong Th1 cells. Alternative populations of T cells stimulated by RV-infected DC could be Th2 or nonpolarized or regulatory T cells (4, 23, 50). To determine if RV infection of MDC could modulate their capacity to stimulate polyclonal T cells, we used MDC infected with RRV or uninfected and then treated with SEB to stimulate $CD4^+$ T cells (Fig. 5). In addition to evaluating IFN-γ (Th1) and IL-13 (Th2) secretion by activated $CD4^+$ T cells, we also looked for the secretion of IL-10 and IL-2, since these cytokines are secreted by regulatory T cells and a subset of nonpolarized T cells, respectively (4, 54). Similar frequencies of SEB-activated T cells secreting IFN-γ, IL-2, IL-10, and IL-13 were stimulated by MDC infected with RRV or uninfected (Fig. 5). There was no statistical difference between these frequencies ($n = 4$; Wilcoxon test, $P > 0.27$). Thus, infection of MDC with RV does not seem to modify their capacity to stimulate polyclonal T cells.

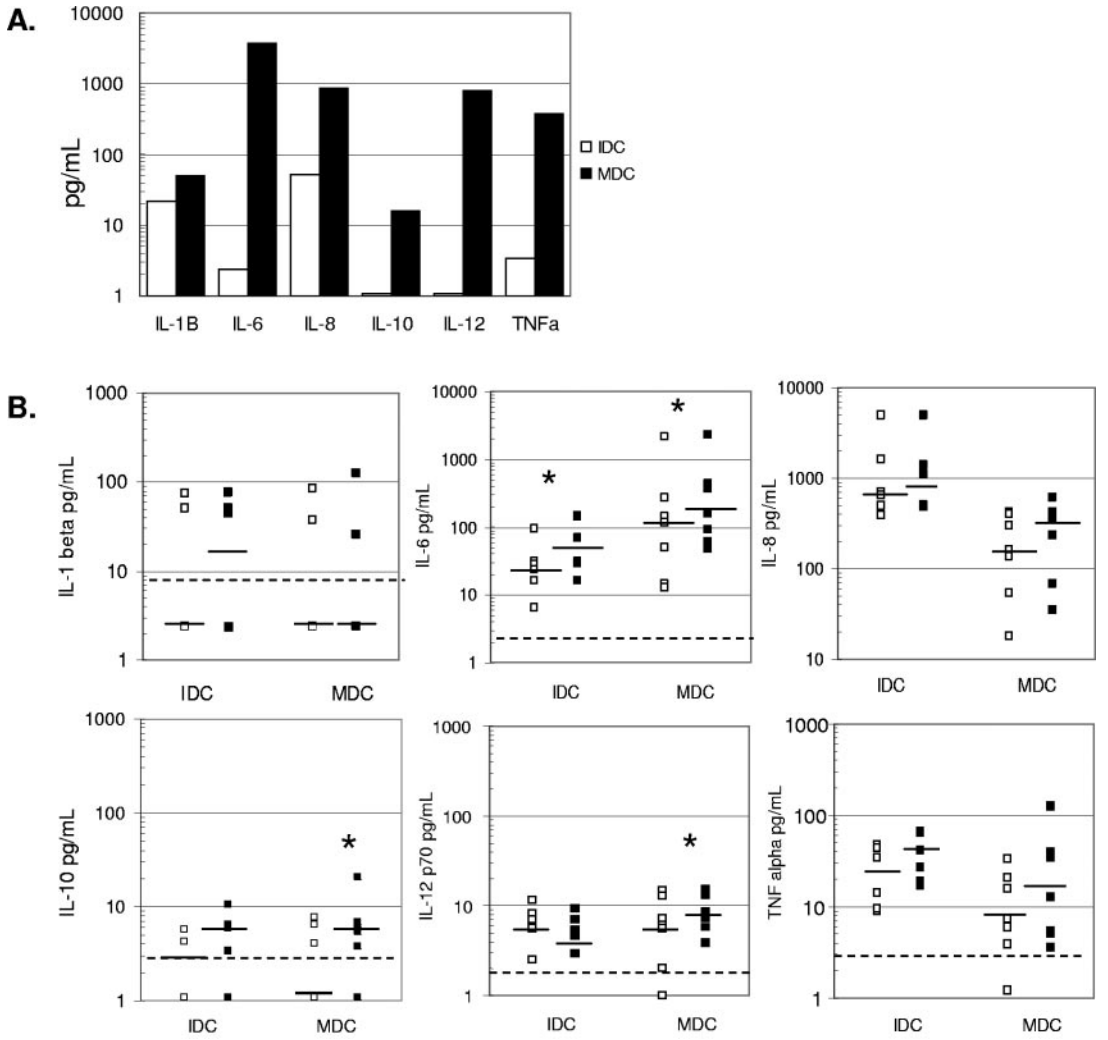

FIG. 4. Cytokines produced by IDC and MDC treated with RV. (A) Cytokines present in the supernatants of IDC and MDC obtained as described in Materials and Methods. The bars represent medians of seven experiments. (B) DC were treated with the control preparation or RV at an MOI of 5 for 48 h. Cytokines in the supernatants of the cultures were measured by CBA. The results of six and seven experiments with IDC and MDC are shown, and the horizontal lines represent medians. The dotted lines in the graphs indicate the level of sensitivity of the assay. *, significant differences between control and RV-treated APC ($P < 0.04$; Wilcoxon test). The limit of detection of IL-8 was 3.6 pg/ml.

The frequency and patterns of cytokines secreted by RV-specific memory CD4$^+$ T cells stimulated by infected MDC are similar to those evidenced after stimulation of PBMC with RV. When PBMC of healthy adults are stimulated in vitro with RV, low frequencies of RV-specific memory T cells that secrete IFN-γ but not IL-4 or IL-13 are evidenced (23, 48). To determine the relative efficiency of RV-infected MDC in activating these T cells, we compared the frequencies and patterns of cytokines produced by memory RV-specific CD4$^+$ T cells after stimulation of PBMC with RRV or MDC infected with RRV. The experiments described above were performed simultaneously and served as a positive control. Figure 6 shows one representative experiment of four performed. The frequencies and patterns of cytokines secreted by RV-specific CD4$^+$ T cells stimulated by both RV and MDC infected with RV were very similar. Specific T cells secreted IFN-γ but not IL-10 (Fig. 6) or IL-13 (data not shown). The net (cells stimulated by RV minus cells stimulated by the control preparation) frequencies of IFN-γ-secreting RV-specific T cells stimulated by RV (median, 0.04; range, 0.03 to 0.07) and RV-infected MDC (median, 0.035; range, 0.02 to 0.05) did not differ statistically (Wilcoxon test, $P = 0.28$). In one of the four individuals studied, small numbers of IL-2-secreting T cells were detected after stimulation with both RV and RV-infected MDC (Fig. 6). In a second individual, the frequency of CD4$^+$ T cells secreting IL-2 was higher after stimulation of T cells with RV-infected MDC than after direct stimulation of PBMC with RV (net frequencies, 0.06 versus 0.01, respectively). In the remaining two individuals, IL-2-secreting T cells were not detected. We conclude that RV-infected MDC are not more efficient APC than those present in PBMC, in revealing IFN-γ-secreting RV-specific memory T cells. Moreover, neither APC stimulates Th2 or regulatory IL-10-secreting RV-specific T cells. Frequencies of RV-specific IL-2-secreting T cells are variable between individuals, and in some volunteers, MDC may reveal a subset of IL-2-secreting virus-specific memory T cells. Similar conclu-

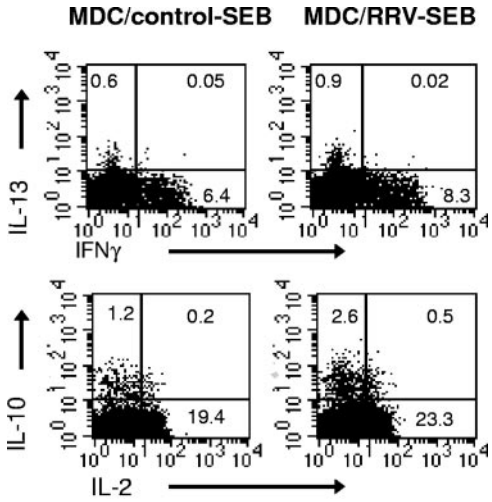

FIG. 5. MDC infected with RV or uninfected stimulate polyclonal $CD4^+$ T cells that secrete similar patterns of cytokines. MDC were treated with control or infected with RV at an MOI of 5 for 24 h. The cells were then incubated with SEB for 30 min, washed, and incubated with autologous PBMC at a 1:10 ratio. The frequencies of $CD4^+$ $CD69^+$ cells expressing intracellular IFN-γ, IL-2, and IL-10 were evaluated. The dot plots shown are gated on $CD4^+$ $CD69^+$ cells. The percentages of cells in the upper and right quadrants are indicated. The results are representative of four experiments performed.

sions were derived from experiments in which PBMC were stimulated with RV-treated IDC ($n = 2$) (data not shown).

IDC treated with RV stimulate allogeneic naive $CD4^+$ T cells to secrete Th1 cytokines. Finally, we sought to determine if RV could modulate the cytokine secretion pattern of naive $CD4^+$ T cells stimulated by IDC. For this purpose, IDC treated with RV, the control preparation, or poly(I · C) (as a positive control) for 24 h were used to stimulate allogeneic naive $CD45RA^+$ $CD4^+$ T cells for 7 days. The pattern of cytokines secreted by the T cells was then evaluated by flow cytometry after stimulation with PMA and A23187 Ca^{2+} ionophore (7). As can be seen in Fig. 7, RV-treated IDC were as efficient as, or more efficient than, IDC treated with poly(I · C) at stimulating naive $CD4^+$ T cells to secrete a Th1 pattern of cytokines. As expected, IDC treated with the control preparation induced nonpolarized T cells or low levels of Th1 cells. Thus, treatment of IDC with RV favors their capacity to stimulate naive $CD4^+$ T cells to secrete Th1 cytokines.

DISCUSSION

We have studied the interaction of RV with human myeloid DC and have shown that (i) RV does not induce apoptosis or DC death, (ii) RV induces maturation of one-fifth of IDC, (iii)

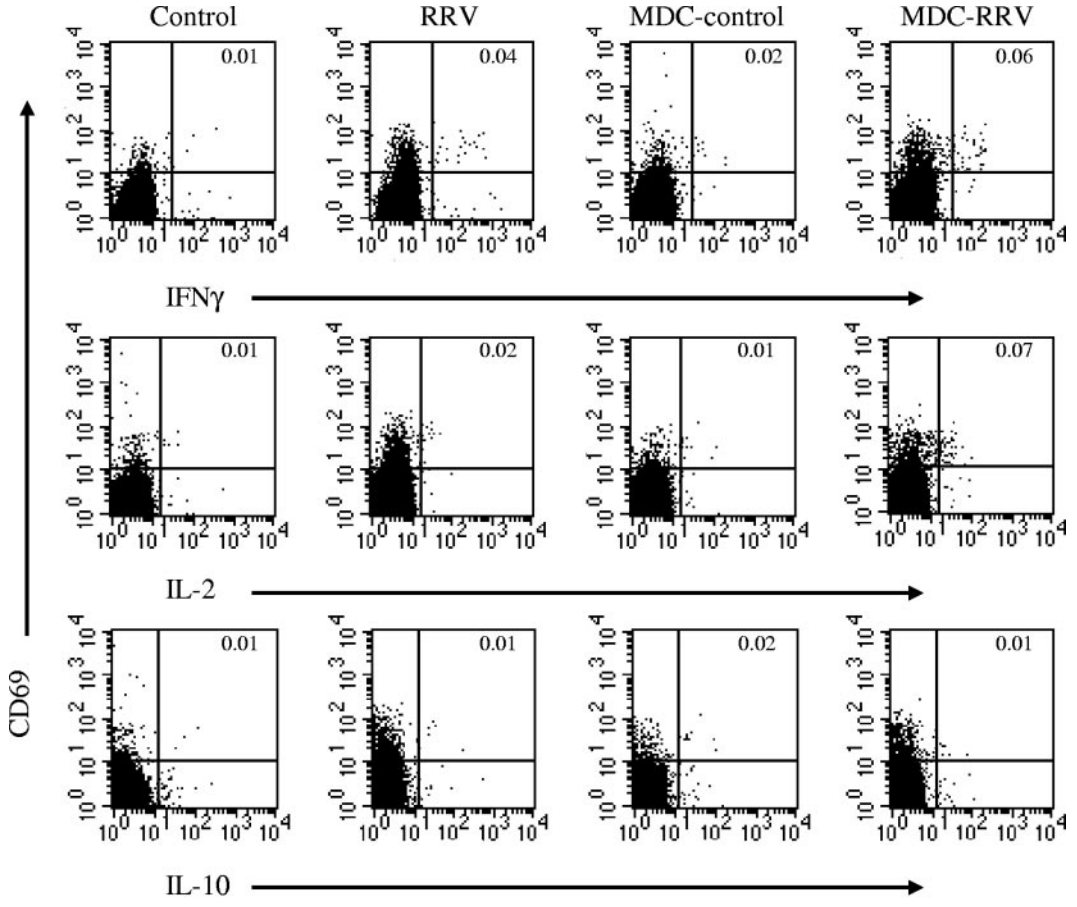

FIG. 6. The frequencies and patterns of cytokines secreted by memory $CD4^+$ T cells evidenced after stimulation of PBMCs with RV or RV-infected MDC are similar. PBMC were stimulated with control, RV, control-treated MDC, or RV-infected MDC. Frequencies of $CD4^+$ $CD69^+$ cells expressing intracellular IFN-γ, IL-2, and IL-10 were evaluated. The dot plots are gated on $CD4^+$ cells. The percentages of cells in the upper quadrants are shown. The results are representative of four experiments performed.

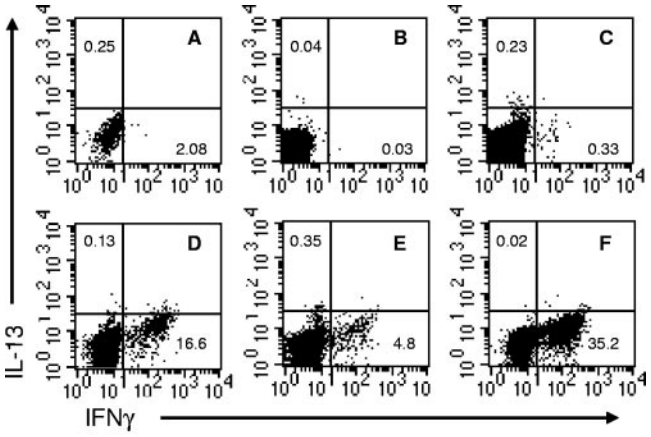

FIG. 7. Naive $CD4^+$ T cells stimulated with RV-treated IDC secrete a Th1 pattern of cytokines. Microbead-purified naive $CD45RA^+$ $CD4^+$ T cells were stimulated with IDC treated with RV or the control preparation. Seven days later, the cells were stimulated with PMA ionophore or unstimulated, and the patterns of cytokines secreted by the T cells were evaluated as in Fig. 5 and 6. Shown are T cells stimulated with (A) poly(I · C)-treated IDC followed by DMSO (PMA and ionophore diluent), (B) medium (no IDC) followed by PMA ionophore, (C) IDC followed by PMA ionophore, (D) poly(I · C)-treated IDC followed by PMA ionophore, (E) control preparation-treated IDC followed by PMA ionophore, and (F) RV-treated IDC followed by PMA ionophore. The dot plots are gated on $CD4^+$ $CD69^+$ cells and show expression of intracellular staining for IFN-γ and IL-13. The percentages of cells in the upper left and lower right quadrants are shown. The results are representative of three experiments performed.

RV does not inhibit maturation of IDC or modulate the phenotype of MDC, (iv) RV preferentially infects MDC, (v) RV induces the production of IL-6 by IDC and MDC and low levels of IL-10 and IL-12p70 by MDC, (vi) RV infection of MDC does not modulate their capacity to stimulate polyclonal T cells, (vii) the frequencies and patterns of cytokines secreted by circulating memory T cells stimulated with RV-infected MDC is similar to those of cells stimulated by RV in PBMC, and (viii) RV-treated IDC induce allogeneic naive $CD4^+$ T cells to secrete Th1 cytokines.

Contrary to what has been shown for a subset of viruses (1, 2, 10, 17), RV does not seem to induce cell death of any of the DC studied (Fig. 1). Like RV, the related reoviruses also do not seem to induce cell death of murine DC (12). Nonetheless, these viruses do not appear to replicate in DC (12). Given that RV is a lytic virus, it seems surprising that it does not induce cell death in MDC in which we have shown a small productive infection. DC have special ways to respond to infection with pathogens; for example, contrary to other cells, upon infection, DC increase the synthesis of cellular proteins, particularly those that enable them to start an immune response (7). Furthermore, DC rapidly upregulate the expression of proteins, such as the MxA protein, that permit them to inhibit the viral cytopathogenic effect (20, 32). Our results suggest that these or similar protective mechanisms are functional in RV-infected MDC. Nonetheless, under the conditions studied there is a basal level of APC death (Fig. 1). For this reason, we cannot rule out minor effects of RV on APC death, especially on the very few potentially infected IDC.

In contrast to poly(I · C) and what has been reported with other viruses, like influenza virus (7), RV did not induce maturation of most IDC. Purified RV dsRNA also induces some degree of maturation of human IDC (37). It is difficult to know if the partial maturation induced by the live viral particle that we have observed is due to the viral dsRNA, since this dsRNA is not free during the viral replication process (15). Further studies are required to determine the pathway by which RV induces maturation of IDC.

Unlike, for example, varicella-zoster virus (38), herpes simplex virus type 1 (28), or cytomegalovirus (CMV) (44), RV did not alter the expression of molecules involved in antigen presentation of MDC. In addition, also unlike herpes simplex virus type 1- (42, 49) and human immunodeficiency virus-infected IDC (17), RV did not inhibit the maturation capacity of the cells (Fig. 1). As opposed to these viruses, which induce persistent infection, RV antigen presentation seems to be sufficient to induce effector cells that mediate viral clearance.

Probably related to the fact that IDC have a higher capacity to endocytose, a tendency exists for viral infection to be favored in IDC over MDC (reviewed in reference 31). This has been documented for human DC interacting with human immunodeficiency virus (17), influenza virus (7), vaccinia virus (10), and herpes simplex virus type 1 (49), among others. In two cases, the opposite seems to be true: respiratory syncytial virus seems to infect CD40L-stimulated myeloid DC more efficiently than IDC (1). Also, some strains of CMV infect mature Langerhans cells better than immature Langerhans cells (19). RV is thus a new example of a virus that favors infection of MDC over IDC and indicates that other factors, in addition to the endocytosis pathway, are important in viral infection of DC.

The inhibition of infection of MDC with an anti-VP7 neutralizing MAb suggests that the mechanisms of entry of RV into MDC and cell lines commonly used to grow RV in vitro are similar. Several integrins have been shown to be involved in the process of RV entry into these cell lines (34). Of the integrins known to be implicated in RV entry, only α4 seems to be preferentially expressed in MDC relative to IDC (43). Future studies will evaluate if the increase in α4 expression in MDC can explain the increased susceptibility of these cells to infection. Also, since RV infected only a fraction of MDC, it will be of interest to see if any particular subset of MDC is preferentially infected.

Most of our studies were done with purified heterologous RRV. However, we also compared the capacity of tissue culture supernatant of homologous Wa RV-infected cells with that of the tissue culture supernatant of RRV-infected cells to induce death and modulate the phenotype and ability to infect IDC and MDC (Fig. 2D and data not shown). In these experiments, the two supernatants were comparable. Moreover, the frequencies of RV-specific memory T cells among PBMC stimulated with Wa tissue culture supernatant or Wa-infected MDC were similar to those obtained after stimulation of PBMC with RRV supernatant or RRV-infected MDC (data not shown). Although experiments with purified Wa RV are needed to confirm these results, our data suggest that homologous RV interact with DC similarly to RRV.

Unlike dsRNA, RV does not induce IDC to secrete detectable amounts of IL-12 (Fig. 4). The poor production of this cytokine by IDC suggests that viral dsRNA is not available to

stimulate toll 3 receptors on IDC (37). The low levels of secretion of IL-12 by RV-infected MDC could be explained by the relative exhaustion of these cells after a potent primary activation stimulus (29). However, both IDC and MDC (exhausted or not) stimulated with CD40L synergistically with other stimuli secrete important amounts of IL-12 (9, 21, 26). In our cultures with naive $CD4^+$ T cells, the presence of CD40L could have stimulated the IDC to produce more IL-12 that could favor the Th1 response we have demonstrated (Fig. 7). The presence of IL-12 has been reported in the supernatants of cultures of simian DC treated with RV and T cells (52).

The frequencies of RV-specific memory T cells secreting IFN-γ evidenced after stimulation of PBMC with RV and infected MDC were very similar (Fig. 6). This was unexpected, since in many models, DC have been shown to reveal higher frequencies of specific T cells than after stimulation with monocytes or direct stimulation of PBMC (13, 33). For this reason, the use of MDC has facilitated the study of T cells specific for several viruses, including influenza and dengue viruses (7, 20, 32). On the other hand, for certain viruses that inhibit the function of DC, like CMV, MDC are not much better at presenting CMV antigen to T cells than monocytes (35). Our results suggest that a similar situation occurs with RV, probably due to the relatively unfavorable pattern of cytokines secreted by the DC. Nonetheless, the efficient stimulation of polyclonal T cells by MDC infected with RV (Fig. 5) suggests that this effect is not a major impediment to their antigen-presenting function. This conclusion is further supported by our findings that treatment of IDC with RV induces these APC to become an efficient stimulus for naive T cells to become Th1 cells (Fig. 7).

Due to the selective replication of RV to enterocytes, the APC that first presents RV antigen in vivo is probably a DC in the intestinal mucosa. However, it has recently been reported that children with acute RV diarrhea have a very important antigenemia early in infection (5). Although infectious virus was not evaluated in blood samples from children, it was detected in the blood samples from infected mice. Presentation of this circulating RV antigen by extraintestinal DC is very likely, and the low levels of RV-specific T cells we have detected (23) cannot be solely attributed to the tolerogenic environment of the intestinal APC (22). We propose that extraintestinal DC, similar to the ones we have studied here, are implicated in the presentation of viral antigen in vivo. In support of this hypothesis, results from a recent study showed that, similar to the cytokines secreted by DC (Fig. 4), there are increased levels of IL-6, IL-10, and IFN-γ but not of IL-1β, IL-2, IL-4, IL-8, IL-12, and TNF-α in the sera of children with RV diarrhea (25). The facts that most IDC are not infected or matured by RV and that RV-treated IDC and infected MDC secrete a pattern of cytokines that does not strongly favor the development of Th1 T cells could partially explain the low levels of circulating RV-specific Th1 cells we have reported previously (23). However, other factors seem to play a role, since IDC treated with RV can efficiently prime a strong allogeneic Th1 response (Fig. 7).

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