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Interplay between Modified Vaccinia Virus Ankara and Dendritic Cells: Phenotypic and Functional Maturation of Bystander Dendritic Cells[▽]

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Modified vaccinia virus Ankara (MVA) is an attenuated poxvirus strain, currently under evaluation as a vaccine vector in various clinical settings. It has been reported that human dendritic cells (DCs) mature after infection with MVA, but reports on the functionality of DCs have so far been controversial. In this work, we studied the phenotype and functionality of MVA-infected DCs. As previously reported, we found that human monocyte-derived DCs upregulated CD86 and HLA-DR in response to MVA infection. Moreover, infected DCs produced a broad array of chemokines and cytokines and were able to activate and induce gamma interferon (IFN-γ) production both in $CD4^+$ and in $CD8^+$ allogeneic T cells and in specific autologous peripheral blood lymphocytes (PBLs). Analysis of DC maturation following infection with a recombinant green fluorescent protein (GFP)-expressing MVA revealed that upregulation of CD86 expression was mainly observed in GFP^{neg} (bystander) cells. While GFP^{pos} (infected) DCs produced tumor necrosis factor alpha (TNF-α), they were unable to produce CXCL10 and were less efficient at inducing IFN-γ production in CEF-specific autologous PBLs. Maturation of bystander DCs could be achieved by incubation with supernatant from infected cultures or with apoptotic infected cells. Type I IFNs were partially responsible for the induction of CXCL10 on bystander DCs. Our findings demonstrate for the first time that, in MVA-infected DC cultures, the leading role with respect to functionality and maturation characteristics is achieved by the bystander DCs.

Attenuated poxviruses are currently being developed and evaluated as vaccine vectors against various infectious diseases, such as HIV infection/AIDS, malaria, tuberculosis, and some types of cancer (22). Vaccinia virus (VV), a member of the poxvirus family with considerable homology to smallpox and cowpox, has been used as a vaccine to successfully eradicate human smallpox. Although considered highly safe in humans, vaccination with VV strains can cause significant and fatal complications, especially in immunocompromised hosts and young children (27). This is partly due to the fact that VV encodes an enormously variable set of immune evasion mechanisms in its large genome (~200 kb), including interactions with complement, cytokines, chemokines, and cell signaling (42). Modified vaccinia virus Ankara (MVA) is a highly attenuated strain, developed by serial passage in primary chicken embryo fibroblasts to serve as a safer vaccine against smallpox (45). After more than 570 passages, the MVA genome suffered 6 major deletions and various gene fragmentations, leading to the loss of the broad cellular host range of its parental strain as well as several genes related to modulation of apoptosis and the immune response of the host (1) which contributed to increasing the virus safety profile, without affecting its abilities to express high levels of viral and foreign proteins and to induce cellular immune responses. These characteristics made it an interesting candidate for the development of vaccines in settings where a T-cell response is required to control disease.

Immunization with VV replicative strains, such as the Dryvax vaccine strain Wyeth or Western Reserve, as well as with attenuated MVA, induces antibody production as well as $CD4^+$ and $CD8^+$ T-cell responses, both in humans (34) and in mice models (5, 47). However, when VV interaction with human dendritic cells (DCs) was studied *in vitro*, VV was found to inhibit maturation of these cells and severely reduced their ability to induce proliferation of naïve T cells (19). Maturation of DCs is a fundamental step before the generation of an adaptive immune response. Since VV is highly immunogenic, it would be expected to induce maturation of DCs *in vivo*, and in fact, this has been demonstrated to occur in a mouse model (48). Unlike VV, and as a consequence of attenuation, MVA was shown to induce maturation of human DCs *in vitro*, evidenced as moderate increase in cell surface CD80 and CD83, a more marked increase in CD86, and production of low levels of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) (17). However, infection of immature human DCs with MVA at a high multiplicity of infection (MOI) impaired their ability to upregulate costimulatory molecules after lipopolysaccharide (LPS) stimulation, a classical maturation signal (26). Moreover, although MVA was also shown to induce DC mat-

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uration *in vivo* in mice, *in vitro* infection of bone marrow-derived DCs with MVA reduced their ability to stimulate allogeneic T cells as well as the level of major histocompatibility complex (MHC) class I molecule expression (4). Given the potential value that MVA holds as a promising vaccine vector, it is clear that more research is needed on how MVA interacts with DCs, what mechanisms are involved in their maturation of DCs, and how these cells generate a potent T-cell response to the vector.

In this work, we have addressed this issue and demonstrated that MVA induced maturation of human monocyte-derived DCs and the production of a large array of cytokines and chemokines. The increase in costimulation was accompanied by an increased ability of these DCs to stimulate allogeneic $CD4^+$ and $CD8^+$ and syngeneic antigen-specific $CD8^+$ T cells. However, by use of a green fluorescent protein (GFP)-expressing virus, we found that at 24 h postinfection (p.i.), maturation was mainly observed in the noninfected bystander DCs of the cultures. Although MVA-infected DCs could still produce TNF-α, bystander DCs produced large amounts of IP-10/CXCL10, a T-cell-activating chemokine, and efficiently presented Ag to CEF-specific syngeneic T cells, inducing IFN-γ production. Bystander DC maturation involved both soluble mediators and infected cells. Finally, type I IFNs participated in IP-10/CXCL10 induction and partially in DC maturation. These data fit with previous *in vivo* works using mice, which showed that induction of $CD8^+$ T-cell responses against MVA-encoded antigens were dominated by cross-priming and suggest that a similar scenario could be taking place after MVA immunization in humans.

MATERIALS AND METHODS

Virus stocks. The wild-type strain of MVA used in this work was the clonal isolate MVA-F6, obtained after 582 passages in CEF cells (44), kindly provided by G. Sutter. MVA expressing the GFP under the natural VV early/late promoter P7.5 has been described previously (20). Stocks were grown on BHK-21 cells, and viruses were released from the infected cells by several rounds of sonication and then purified by centrifugation through a sucrose cushion and titrated by immunostaining in BHK-21 cells using a rabbit polyclonal antibody against VV antigens.

Generation of human DCs. Buffy coats from healthy donors were prepared at Sanatorio Julio Mendez (Buenos Aires, Argentina) according to institutional guidelines. Informed consent was obtained from each donor before blood collection. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll gradient separation on Ficoll-Paque (GE Healthcare). Then, monocytes were purified by centrifugation on a discontinuous Percoll gradient (GE Healthcare). Monocytes were recovered at the 56-to-46% interface. Peripheral blood lymphocytes (PBLs) were obtained from the 62-to-56% interface and frozen in a mixture of 90% fetal calf serum (FCS) plus 10% dimethyl sulfoxide (DMSO) at −80°C for 5 to 6 days, to be used later in cocultures. Purity was checked by flow cytometry for both populations and was found to be >85% and >90% for monocytes and PBLs, respectively. To obtain DCs, monocytes were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal calf serum (complete medium; all from Invitrogen) at 10^6 cells/ml with 10 ng of IL-4/ml (BD Biosciences) and 20 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF)/ml (Sigma). On day 5 or 6, differentiation and maturation status were analyzed by staining with fluorochrome-conjugated antibodies against human CD1a, CD14, and CD86 (BD Biosciences).

Infection of immature DCs. DCs were infected with wild-type MVA or MVA-GFP at different MOIs (from 0.1 to 4), depending on the experiment. For this, 10^6 cells were placed in polypropylene tubes and resuspended in 50 μl of RPMI 1640 without fetal bovine serum (FBS) containing the virus for 90 min. To avoid adhesion to the tube and equal distribution, the tubes were gently shaken every 15 min. After this, cells were washed 3 times with phosphate-buffered saline (PBS) to remove excess virus, resuspended in complete medium at a density of 2×10^6 cells/ml, and seeded in 24-well flat-bottom culture plates in a total volume of 1 ml. In some experiments, DCs were also treated with 100 ng/ml LPS (from *Escherichia coli* 0111:B4 strain; Sigma) as positive controls. Supernatants were recovered at 24 h p.i., centrifuged, and stored at −80°C until use. Viability of infected cells was assessed by trypan blue exclusion before every experiment.

For some experiments, infection was performed at an MOI of 5, and after 24 h, supernatants and infected cells were recovered and centrifuged at $400 \times g$ for 10 min. Then, supernatants were filtered with a 0.22-μm syringe filter to remove large debris and some of the remaining virus (the size of VV virions is approximately 0.26 to 0.34 μm by 0.32 to 0.38 μm) (33). Infected cells were washed 3 times in PBS to remove loosely attached virions and resuspended in complete medium. The number of viable cells was estimated by a trypan blue exclusion assay, and apoptosis was assessed by annexin V-propidium iodide staining (fluorescein isothiocyanate [FITC] annexin V apoptosis detection kit I; BD Biosciences), according to the manufacturer's instructions. In some cases, viral gene expression from the remaining virus in the infected apoptotic cells was prevented by UV treatment (5 min using a 365-nm UV bulb positioned 8 cm over the sample). The amount of infective virus in supernatants and infected cells and the effect of UV treatment were assessed by immunostaining in BHK-21 cells.

Mixed lymphocyte culture. The allogeneic mixed lymphocyte reaction (MLR) was used to monitor DC function. DCs were infected with MVA and mock treated or matured with 100 ng/ml LPS, and 24 h later, they were washed, counted with trypan blue, and fixed in PBS containing 0.5% paraformaldehyde for 15 min at 4°C. Then, cells were washed twice with PBS, resuspended in complete medium, and added in graded doses to 2×10^5 allogeneic PBLs (DC/PBL ratios from 1:25 to 1:400) in 96-well flat-bottom plates (Greiner Bio-One) in triplicate. Proliferation was determined on day 4 with the addition of 1 μCi/ml of [^{3}H]thymidine during the last 16 h. Then, cells were harvested, and measurement of incorporated thymidine was performed with a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer). Supernatants were obtained from parallel cultures, centrifuged, and stored at −80°C until use.

To study T-cell activation during DC-PBL coculture, DCs were treated as before, incubated for 24 h, and then irradiated (3,000 rads) and incubated with allogeneic PBLs at a 1:20 ratio in 24-well flat-bottom plates (Greiner Bio-One). IFN-γ production was analyzed after 3 days by intracellular cytokine staining. Upregulation of maturation markers was studied at day 5 of coculture by flow cytometry.

Flow cytometry. The following antibodies were used to study surface expression of maturation markers on DCs and activation markers on T cells: phycoerythrin (PE)-labeled anti-CD83 (clone HB15e), PE-labeled anti-CD86 (clone FUN-1), PE-labeled anti-HLA-DR (clone L243), FITC-labeled anti-HLA-ABC (clone G46-2.6), peridinin chlorophyll protein (PerCP)-labeled anti-CD3 (clone SK7), allophycocyanin (APC)-labeled anti-CD4 (clone SK3), APC-Cy7-labeled anti-CD8 (clone SK1), PE-labeled anti-CD38 (clone HB7), and FITC-labeled anti-CD69 (clone L78). In all cases, isotype-matched control antibodies were used. All antibodies were purchased from BD Biosciences (Argentina). DCs were stained at 24 h p.i.; distinction between MVA-infected and noninfected bystander DCs was done through GFP intensity. T cells from cocultures were stained after 5 days. At each time point, cells were washed with cold PBS and stained with the directly conjugated antibodies for 30 min at 4°C in 50 μl of PBS. Cells were washed twice and resuspended in 200 μl cold PBS. Samples were acquired on a FACSCanto cytometer and analyzed by FACSDiva software (BD Biosciences, Argentina).

Measurement of cytokine production. Supernatant from MVA-infected DC cultures or mixed lymphocyte cultures were diluted with complete medium and used for the simultaneous determination of 48 cytokines/chemokines with Bio-plex human cytokine group I (27 cytokines) and Bio-plex human cytokine group II (21 cytokines) (Bio-Rad Laboratories, CA), using Luminex technology as previously described (21).

Intracellular cytokine staining. IFN-γ production in T cells from DC-PBL cocultures was analyzed after 3 days. For this, brefeldin A (1 μl/ml of GolgiPlug; BD Biosciences) was added for the last 12 h of the culture. Then, cells were harvested, washed in PBS, and stained with fluorochrome-conjugated antibodies against CD3, CD4, and CD8 as stated in "Flow cytometry." Cells were fixed and permeabilized according to the manufacturer's instructions (Cytofix/Cytoperm kit; BD Biosciences). After being washed twice in the supplied buffer, cells were intracellularly stained with antibodies against PE-labeled human IFN-γ (clone B27; BD Biosciences). Cells were subsequently washed in the supplied buffer and stored at 4°C in the dark until flow cytometric analysis (performed within 24 h).

Production of TNF-α and IP-10/CXCL10 in MVA-infected, mock-treated, or LPS-matured DCs was assessed at 20 h p.i. Brefeldin A was added as before for the last 12 h of the culture. Then, cells were harvested and stained for the

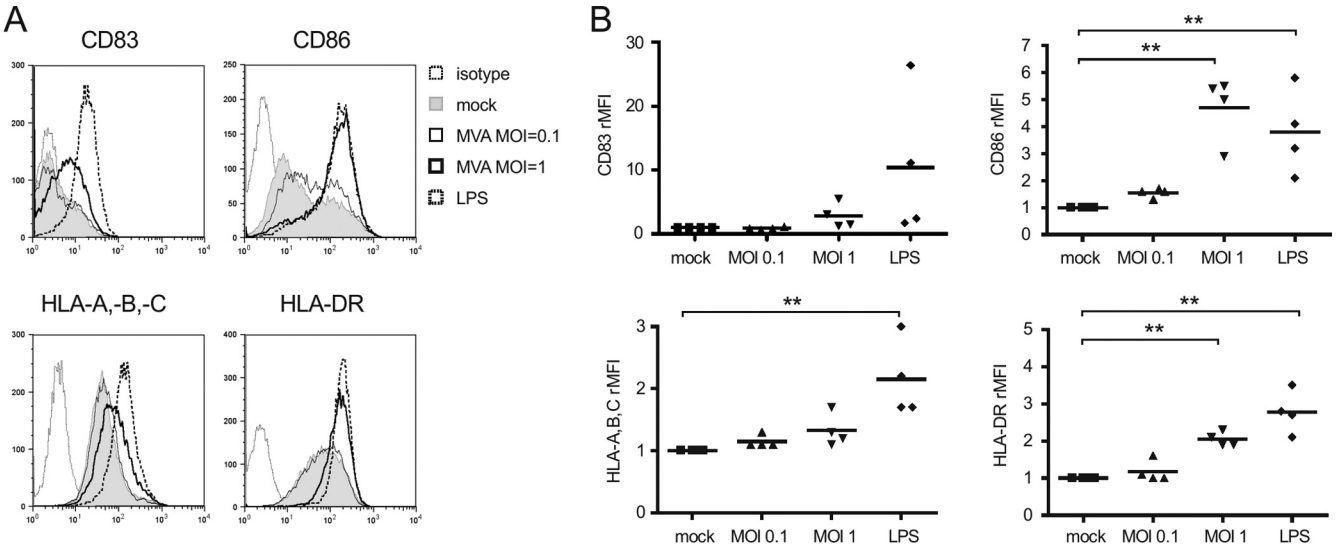

FIG. 1. MVA induces phenotypic maturation of immature DCs. DCs were either infected with MVA at different MOIs (0.1 and 1), incubated with 100 ng/ml LPS, or mock-infected and then cultured for 24 h. Cell surface expression of CD83, CD86, HLA-A, -B, and -C, and HLA-DR was analyzed by flow cytometry. (A) A representative histogram is shown for each condition and marker. (B) Results are depicted for 4 different donors as the relative mean fluorescence intensity (rMFI), calculated as [(MFI for sample − MFI for isotype)/(MFI for mock infection − MFI for isotype)]. The average rMFI value is shown for each condition and marker. Statistical differences between groups are also shown for each marker (**, $P < 0.01$).

intracellular mediators as stated above. In this case, PE-labeled anti-TNF-α (clone MAb11; BD Biosciences) and PE-labeled anti-IP-10 (clone 6D4/D6/G2; BD Biosciences) antibodies were used. Distinction between MVA-infected and noninfected bystander DCs was done through GFP intensity.

CEF-specific ELISPOT assay. IFN-γ releasing cells were detected upon specific peptide stimulation using an enzyme-linked immunospot (ELISPOT) assay. DCs were differentiated and then infected with MVA on day 5 or 6. On the same day, autologous PBLs were thawed and rested overnight. Polyvinylidene difluoride (PVDF) microplates (96 well, MultiScreen$_{HTS}$-IP filter plates; Millipore) were coated with an antibody against human IFN-γ (human IFN-γ ELISPOT pair; BD Biosciences) overnight at room temperature (RT). At 24 h p.i., DCs were recovered, washed with PBS, and counted with trypan blue. Then, 6×10^4 viable cells were charged with 2 μg/ml CEF peptides (a mixture of peptides from cytomegalovirus [CMV], Epstein-Barr virus [EBV], and influenza virus; NIH AIDS Research and Reference Reagent Program) for 1 h at 37°C, washed twice to remove excess peptides, and placed in coated ELISPOT plates together with 2×10^5 autologous PBLs/well at different ratios in triplicate. Mock-treated and LPS-matured DCs were included as negative and positive controls, respectively. After 20 h, spots were revealed with a biotin-conjugated antibody against human IFN-γ (human IFN-γ ELISPOT pair; BD Biosciences), followed by peroxidase-labeled streptavidin and AEC substrate (BD Biosciences). Plates were scanned on an ImmunoSpot reader (Cellular Technology, Ltd.), and specific spots were counted using the ImmunoSpot software program.

Neutralization of type I IFNs. To neutralize the activity of type I IFNs, specific blocking antibodies against the common receptor IFNAR (clone MMHAR-2; PBL Interferon Source) were added to DCs at a concentration of 50 μg/ml before infection with MVA or supernatant stimulation (15 min at 37°C) and were left in the supernatants until the end of the respective experiment at a concentration of 10 μg/ml. Mouse IgG2a (clone MOPC-173; BD Biosciences) was used as an isotype control.

Statistical analysis. All statistical comparisons were performed with *t* tests or analysis of variance (ANOVA) followed by Dunnett's posttest, as appropriate. *P* values of less than 0.05 were considered statistically significant.

RESULTS

MVA infection induces a distinctive maturation profile in monocyte-derived DCs. A previous report indicated that infection of monocyte-derived DCs with MVA led to a moderate increase in cell surface CD80 and CD83, a more marked increase in CD86, and low levels of TNF-α and IL-6 secretion (17). Additionally, in a recently published paper by Brandler et al., an MVA vector expressing HIV genes was shown to induce upregulation of maturation markers to an extent similar to that induced by LPS (8). Our first aim was to extend these observations, examining the effect of MVA infection on both the expression of costimulatory and MHC molecules and the production of chemokines and cytokines in order to characterize in more depth the maturation profile induced by MVA on DCs. Of note, since VV infection of DCs *in vivo* is estimated to occur at a low MOI (48), we decided to find the lowest MOI at which maturation of DCs still occurred in this *in vitro* setting.

For this, DCs were infected with MVA at increasing MOIs (0.001, 0.01, 0.1, 1, and 5), and then cell surface expression of CD83, CD86, and class I (HLA-A, -B, and -C) and class II (HLA-DR) MHC molecules was analyzed by flow cytometry at 24 h postinfection (p.i.). As a positive control, DCs were incubated with 100 ng/ml LPS during the same period of time. MVA infection led to a significant increase in CD86 and HLA-DR expression and a less marked increase in CD83 and class I molecule expression (Fig. 1A and B). This increase was observed only at an MOI of 1; while a lower MOI did not increase surface marker expression significantly, infection at an MOI of 5 for 24 h led to extensive cell death (Fig. 1A and B and data not shown).

In human macrophages, MVA has been shown to induce robust production of chemokines and a fairly weak inflammatory response (15). However, a similar analysis had not been performed yet for DCs. Therefore, we studied the profile of cytokines and chemokines released by MVA-infected DCs by Luminex technology in supernatants obtained 24 h after infection. An important increase was found for 13 out of the 40 mediators analyzed, while 18 other mediators showed a more

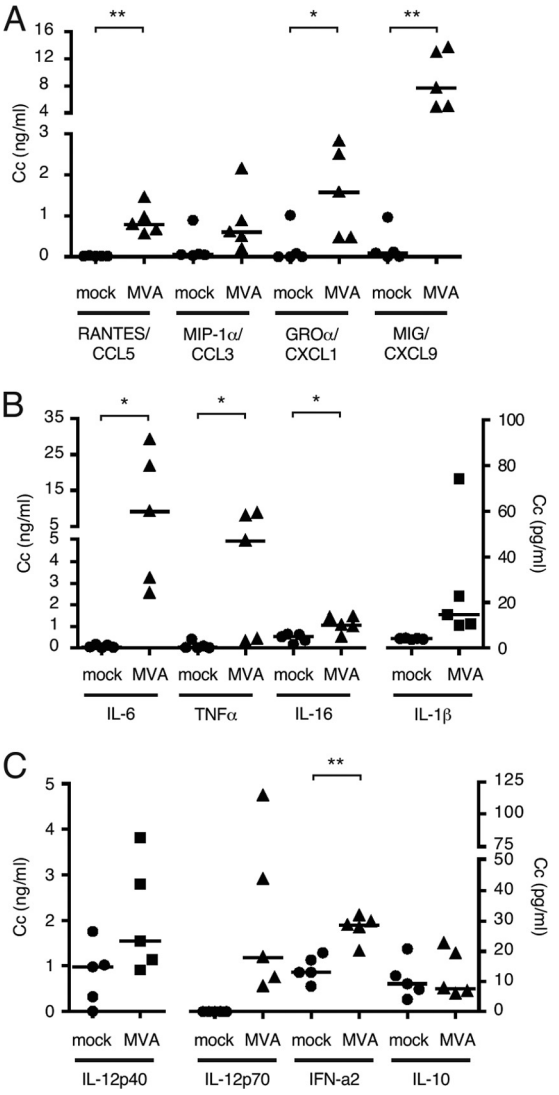

FIG. 2. MVA infection of immature DCs induces a characteristic pattern of cytokines and chemokines. Supernatant from MVA-infected (MOI = 3) or mock-infected DCs was collected at 24 h p.i., centrifuged to pellet cells, and frozen at −80°C until further use. Then, samples from 5 different donors were thawed, and an array of mediators was assayed by Luminex technology. Results are presented for chemokines (A), proinflammatory mediators (B), and T-cell polarizing cytokines (C) as the concentration of mediator found on each supernatant in ng/ml (left *y* axis for IL-6, TNF-a, and IL-16 [A and B] and left *y* axis for IL-12p40 [C]) or pg/ml (right *y* axis for IL-1b [B] and right *y* axis for IL-12p70, IFN-a2, and IL-10 [C]), as appropriate. Statistical differences between levels for MVA-infected and mock-infected DCs are shown (*, $P < 0.05$, and **, $P < 0.01$).

modest increase. High levels of production were observed in 7 out of 12 chemokines, including RANTES/CCL5, MIP-1α/CCL3, GROα/CXCL1, and MIG/CXCL9 (Fig. 2A and Table 1). Remarkably, the levels of IL-8/CXCL8, IP-10/CXCL10, and MIP-1β/CCL4 were above their respective detection limits in 5/5, 4/5, and 4/5 samples from different donors, respectively. On the other hand, only 4 out of 27 cytokines analyzed were produced in high levels in response to MVA. With regard to proinflammatory mediators, IL-6 and TNF-α were found to be present in the supernatants from infected cells at high concentrations, together with IL-16 (Fig. 2B). The last proinflammatory cytokine has been attributed with functions such as chemotaxis of $CD4^+$ T cells, monocytes, and eosinophils, expression of proinflammatory molecules, and activation of antigen-presenting functions in monocytes (12). In addition, MIF concentrations in supernatants from MVA-infected DCs were higher than those found for mock-treated cells and above the limit of detection in 4 of 5 samples (Table 1). GM-CSF was also significantly increased. Concerning T-cell polarizing cytokines, IL-12p70 and IFN-α2 levels were increased after MVA infection, albeit at low levels, while IL-10 production was not induced (Fig. 2C). Other cytokines increased modestly after infection (Table 1).

The results presented in this section show that MVA infection induces a distinctive maturation pattern in human DCs, with strong upregulation of CD86 and HLA-DR, production of high levels of chemokines, and some proinflammatory mediators, together with intermediate levels of Th1-inducing cytokines. This pattern is different from the classical maturation pattern induced by LPS (Fig. 1 and data not shown), which could be related to the fact that MVA activates DCs through TLR2 and intracellular recognition receptors (15, 50). Additionally, a very similar chemokine/cytokine production pattern has been described to occur after DC infection with dengue

TABLE 1. Chemokine and cytokine production by MVA-infected DCs[a]

Mediator	Median (range) (pg/ml) for DCs (n = 5)	
	Mock infected	MVA infected
Chemokines		
MCP-1/CCL2	266 (17–494)	822 (633–3,411)
MIP-1β/CCL4	480 (335–868)	NA (1,717–ADL)
MCP-3/CCL7	16 (2–47)	97 (11–1,109)
Eotaxin/CCL11	0 (0–0)	14 (3–20)
CTACK/CCL27	0 (0–0)	11 (5–28)
IL-8/CXCL8	3,058 (2,555–3,223)	ADL
IP-10/CXCL10	278 (75–4,957)	NA (5,016–ADL)
SDF-1α/CXCL12	21 (8–36)	61 (53–107)**
Cytokines		
GM-CSF	37 (3–6,005)	2,411 (459–11,439)
IFN-γ	28.1 (0.0–62.8)	199.8 (128.6–259.3)**
IL-1α	1.6 (1.3–1.7)	12.3 (6.9–38.8)*
IL-3	0.0 (0.0–1.8)	24.6 (16.8–50.5)
IL-4	41.2 (1.9–119.6)	160.0 (33.0–297.0)
IL-5	0.0 (0.0–0.0)	0.0 (0.0–0.0)
IL-7	0.0 (0.0–0.0)	0.0 (0.0–0.8)
IL-9	18.9 (4.2–23.8)	40.9 (31.9–51.6)**
IL-13	0.0 (0.0–0.0)	0.0 (0.0–2.4)
IL-15	0.0 (0.0–2.5)	1.7 (0.2–7.1)
IL-17	0.0 (0.0–15.2)	25.6 (15.3–34.3)**
IL-18	1.2 (0.8–1.3)	2.9 (1.8–7.8)
IL-1Ra	4,812 (2,739–15,956)	3,930 (2,358–12,961)
IL-2Ra	0.6 (0.0–2.8)	16.3 (7.0–43.7)*
LIF	0.4 (0.0–2.6)	3.7 (0.4–9.3)
MIF	9,446 (2,322–14,711)	35,684 (17,641–56,260)**
SCF	3.7 (3.2–7.3)	19.1 (15.2–41.5)**
TNF-β	1.0 (0.7–9.1)	5.8 (4.6–42.1)*
TRAIL	1.8 (0.0–5.4)	51.4 (27.9–75.1)**

[a] Supernatants from mock-treated or MVA-infected DCs were harvested at 24 h p.i., and mediator levels were measured by Luminex technology. ADL, above detection limit; NA, not applicable. Statistical difference from the value for mock-infected cells is shown (*, $P < 0.05$; **, $P < 0.01$).

virus (35), which suggests that this may be common pattern for viral infections.

MVA-infected DCs are able to activate and induce IFN-γ production both in $CD4^+$ and in $CD8^+$ T cells. It has been described that, as part of their immune evasion mechanisms, replicative strains of vaccinia virus inhibit the maturation of DCs and thus their ability to stimulate the proliferation of allogeneic T cells (19). As reported before (8, 17, 32) and confirmed here, MVA is able to induce maturation of DCs; however, the ability of MVA-infected DCs to activate T cells remains controversial. Drillien et al. found that human DCs infected directly with MVA did not induce a significant number of IFN-γ-producing T cells in a syngeneic ELISPOT assay (17). In addition, Behboudi et al. reported that MVA-infected murine DCs showed impairment in their ability to induce proliferation of allogeneic T cells in an MLR (4). This reported dysfunctionality did not correlate with the mature phenotype, so we next sought to examine the ability of MVA-infected DCs to induce T-cell activation.

For this, we first studied the ability of MVA-infected DCs to induce the proliferation of allogeneic lymphocytes in mixed lymphocyte cultures. MVA-infected (MOI = 1), LPS-matured, or mock-treated DCs were cocultured with allogeneic PBMCs at different ratios, and then proliferation was assessed by [^{3}H]thymidine incorporation. We found that MVA-infected DCs were as efficient as LPS-matured DCs, and more efficient than mock-treated cells, at inducing proliferation of PBMCs (Fig. 3A). The cytokine production profile in supernatants from cocultures after 5 days was then analyzed by Luminex technology. Coculture of PBMCs with MVA-infected DCs led to a great increase in the production of IFN-γ, which reached concentrations of 59 to 109 ng/ml. We also found a smaller induction of IL-17 production in the pg/ml range. Recent reports documented a role for IL-17-producing cells in anti-VV immunity, indicating that Tc17 cells were generated during a vaccinia virus infection and could promote anti-VV immunity (49). On the other hand, Th2-promoting cytokines such as IL-4, IL-5, and IL-13 were not detected; moreover, synthesis of IL-5 and IL-13 was decreased compared to that produced by cocultures of mock-treated DCs and PBMCs (Fig. 3B). These results suggest that MVA-infected DCs are able to induce proliferation and Th1 differentiation of allogeneic T cells.

Immunization of mice and humans with MVA is known to induce both specific $CD4^+$ and $CD8^+$ T-cell responses *in vivo* (16, 34). When we examined the expression of the activation markers CD38 and CD69 on T cells from mixed cultures, we found that coculture with MVA-infected DCs led to increases in $CD38^+$ $CD69^+$ cells both on $CD3^+$ $CD4^+$ and on $CD3^+$ $CD8^+$ populations with respect to coculture with mock-treated DCs (Fig. 3C). Additionally, MVA-infected DCs were able to induce IFN-γ production in both populations of allogeneic T cells, as assessed by flow cytometry (Fig. 3D).

In order to ascertain whether MVA-infected DCs were able to present peptides to and activate antigen-specific T cells to produce IFN-γ, DCs were infected with MVA at the lowest MOI that induced maturation (MOI = 1), incubated for 24 h, and then charged with CEF peptides. This is a mixture of $CD8^+$-restricted peptides that represent epitopes from CMV, EBV, and influenza virus and has been described to be recognized by 88% of healthy Caucasian donors in an ELISPOT assay (13). MVA-infected CEF-charged DCs were placed in contact with syngeneic PBLs at different ratios in an ELISPOT plate, and IFN-γ production was detected after 20 h. As can be seen in Fig. 3E, MVA-infected DCs were as efficient as LPS-matured DCs, and more efficient than mock-treated DCs, at inducing IFN-γ production by CEF-specific $CD8^+$ T cells. Specificity was confirmed since no IFN-γ-producing cells were detected after incubation with DCs that had not been charged with the peptides (data not shown). Together, these results suggest that the phenotypic maturation observed in MVA-infected DCs is also functional and that these DCs are able to activate and induce production of IFN-γ both by allogeneic $CD4^+$ and $CD8^+$ T cells and by virus-specific memory/effector $CD8^+$ T cells.

MVA-infected and bystander DCs present phenotypic and functional differences. Although MVA has lost many immunoregulatory genes, it is still able to rapidly block cellular protein synthesis and induce apoptosis of the infected antigen-presenting cells (10, 26). Moreover, infection of human DCs with MVA at a high MOI blocked LPS-induced maturation (26), suggesting that MVA-infected DCs would not be fully functional. Thus, we hypothesized that the maturation observed at an MOI of 1 could be mainly occurring in the noninfected bystander DCs in the culture. Our next goal was to analyze the maturation statuses of both infected and bystander cells after of 24 h of infection with MVA. To do this, DCs were infected with a recombinant GFP-expressing MVA (MVA-GFP) at different MOIs, and maturation was assessed by flow cytometry in GFP^{pos} (infected) and GFP^{neg} (bystander) cells at 24 h p.i. The efficiency of infection of DCs coming from different donors, evaluated also after 24 h, is shown in Fig. 4A. We found that this proportion was variable, ranging from 2.5 to 26.1% GFP^{pos} cells at an MOI of 1. Remarkably, at this MOI, even when the number of infected DCs was as low as 2.5%, maturation was still observed in the bulk culture (data not shown).

A representative histogram of CD86 and HLA-DR expression for GFP^{pos} and GFP^{neg} cells (MOI = 2) is depicted in Fig. 4B, while Fig. 4C shows the relative mean fluorescence intensity in the two populations as a function of MOI. As can be clearly seen, CD86 and HLA-DR expression levels were regulated differently in GFP^{pos} and GFP^{neg} cells. GFP^{neg} (bystander) DCs showed a significant MOI-dependent upregulation of CD86 and HLA-DR. In the GFP^{pos} (infected) cells, CD86 and HLA-DR levels were slightly increased with respect to those found on the mock-infected cells, but this increase was always much lower than that found for the GFP^{neg} cells in the same culture.

It could be argued that infected DCs could upregulate CD86 early and then die of apoptosis, and thus at 24 h p.i. they would not be detectable anymore. However, we still found a considerable number of viable infected cells at 24 h p.i., which presented low levels of CD86. When kinetic studies were performed, maximal GFP expression was found at 6 h p.i., as described before (10), and then decreased (30.4% at 6 h versus 21.0% at 24 h), which could be attributed to cell death. On the other hand, we cannot rule out the possibility that CD86 expression could increase early in infected cells and then decrease due, for example, to blockade in protein synthesis.

Then, we evaluated whether this dichotomy also extended to

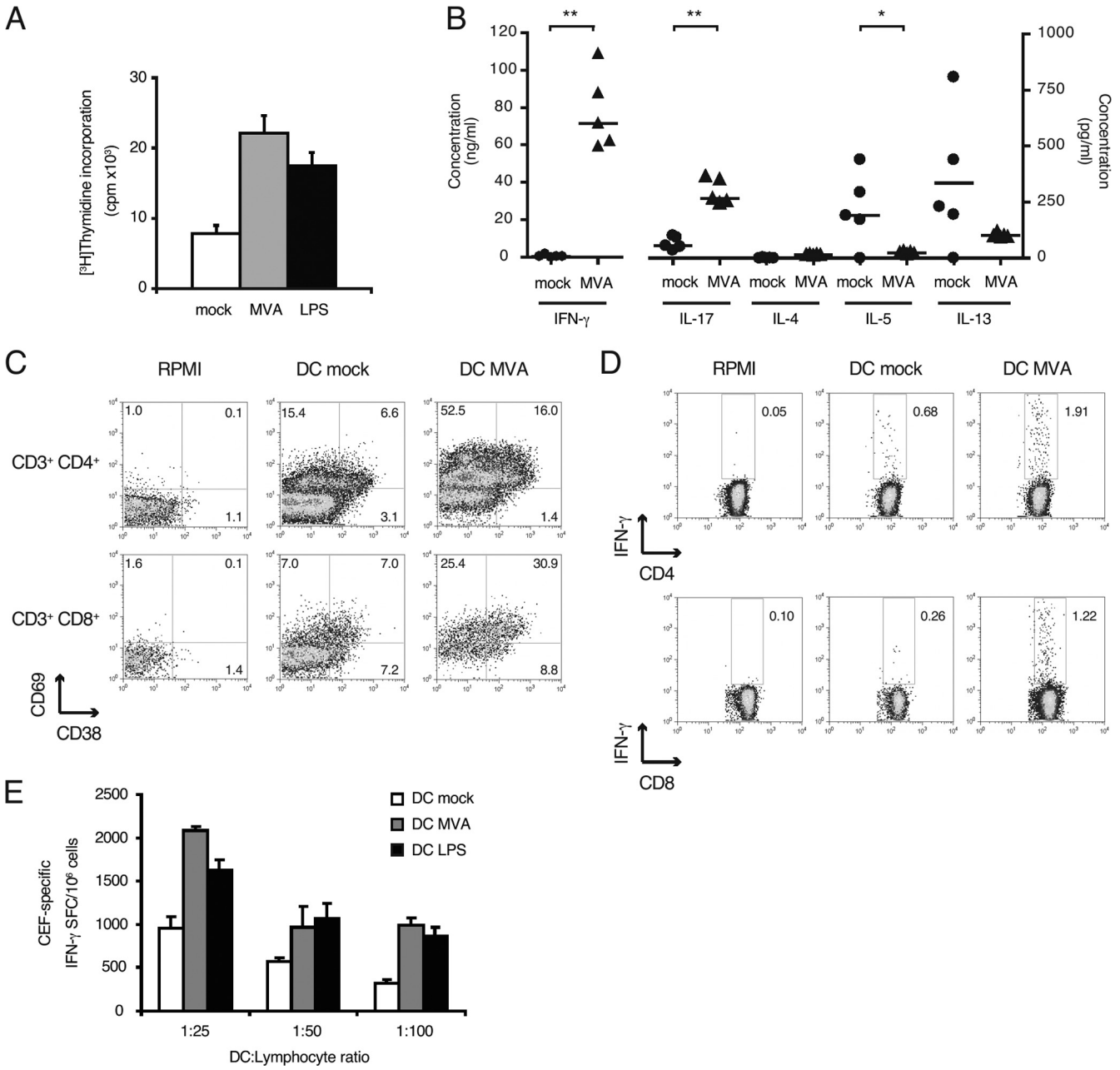

FIG. 3. MVA-infected DCs are functionally mature and efficiently activate $CD4^+$ and $CD8^+$ T cells. (A) DCs were infected with MVA at an MOI of 1 and then washed and left to mature. As controls, DCs were either matured in the presence of 100 ng/ml LPS or mock treated. After 24 h, DCs were harvested, washed, irradiated, and placed in coculture with 2.10^5 allogeneic PBLs at different DC/PBL ratios. Proliferation was assessed by [^{3}H]thymidine incorporation on day 2 of coculture. Results are shown in cpm (mean ± standard deviation [SD]) for triplicate wells of a representative experiment. (B) Cytokines in supernatants obtained after 5 days of allogeneic coculture were measured by Luminex technology. Results are presented as the concentration of mediator found on each supernatant in ng/ml (left *y* axis for IFN-γ) or pg/ml (right *y* axis for IL-17, IL-4, IL-5 and IL-13) ($n = 5$). Statistical difference from the level for mock-infected DCs is shown (*, $P < 0.05$, and **, $P < 0.01$). (C) Staining for activation markers was performed after 5 days of allogeneic coculture. Cells were washed, stained simultaneously with anti-CD3, anti-CD4, anti-CD8, anti-CD38, and anti-CD69 antibodies, and analyzed in a 6-color BD FACSCanto flow cytometer. Graphs show the expression of CD38 and CD69 observed on gated $CD3^+$ $CD8^+$ and $CD3^+$ $CD4^+$ cells after culture in complete medium or with mock-treated (DC mock) or MVA-infected (DC MVA) DCs, and the percentages are specified for each quadrant. (D) IFN-γ expression was analyzed by flow cytometry after 3 days of allogeneic coculture. Brefeldin A was added 12 h before the end of the culture, and then cells were harvested and stained with antibodies against human CD3, CD4, CD8, and IFN-γ. Results are shown as the percentage of IFN-γ^+ cells in gated $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ cells. (E) DCs were infected at an MOI of 1, stimulated with 100 ng/ml LPS, or mock treated for 24 h. Then, they were washed, loaded with CEF peptides, and placed in culture with syngeneic PBLs, at different ratios, on ELISPOT plates coated with an antibody against human IFN-γ. The average number of CEF-specific IFN-γ spot-forming cells (SFC)/10^6 PBLs (± SD; $n = 3$) is depicted for each condition. In panels A, C, D, and E, results for a representative experiment of 3 are shown.

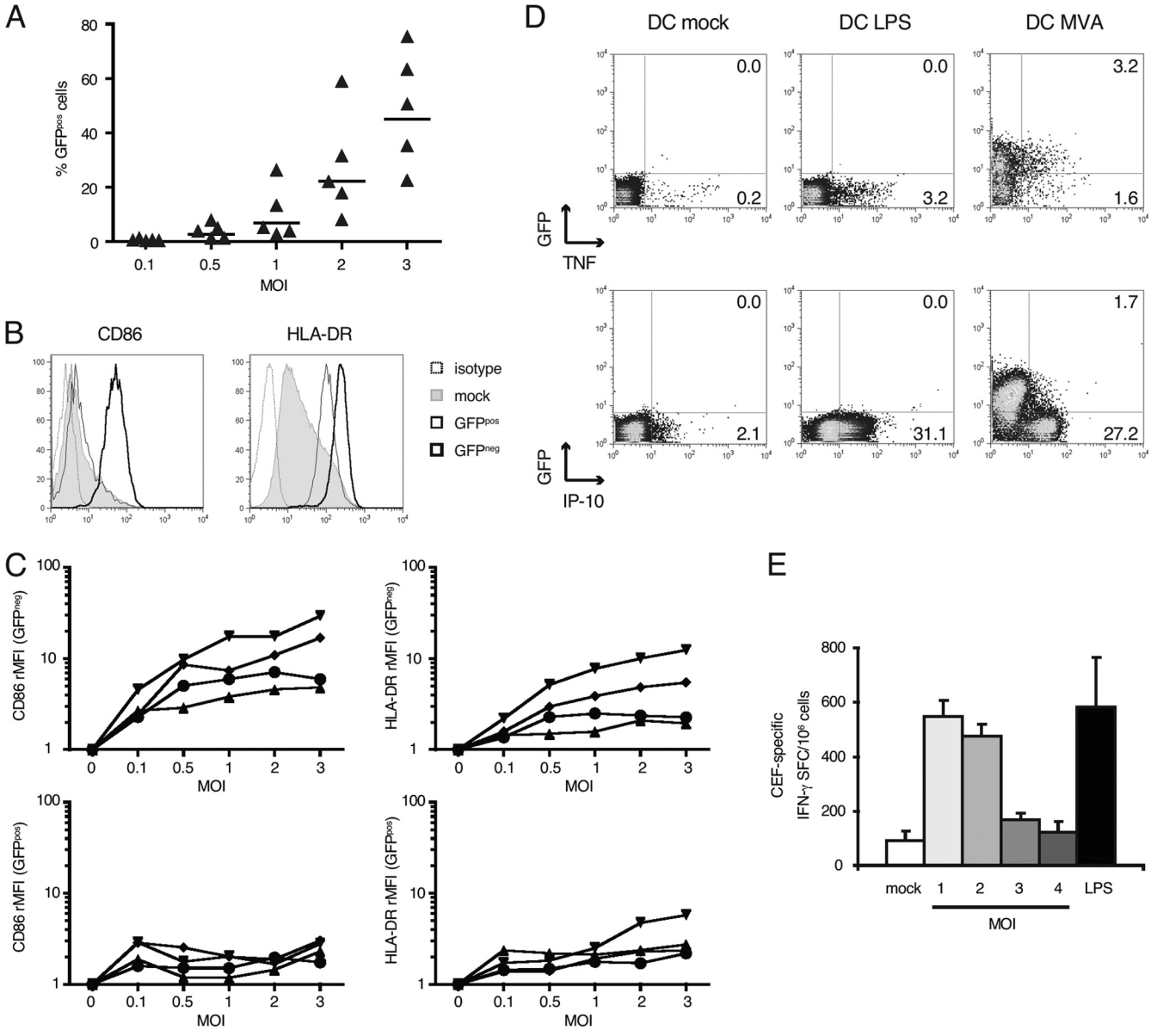

FIG. 4. Differential phenotype and functionality of infected and bystander DCs. (A) DCs were infected with MVA-GFP at different MOIs, and GFP expression was analyzed by flow cytometry at 24 h p.i. Data are expressed as percentages of GFP^{pos} cells for each donor. (B) Expression of maturation markers CD86 and HLA-DR was analyzed for GFP^{pos} (infected cells) or GFP^{neg} (bystander cells) in MVA-infected DC cultures (MOI = 2) or in mock-treated DCs. Matched isotype controls are also included, and a representative set of histograms is presented for each marker. (C) The rMFIs (calculated as described for Fig. 1B) of CD86 and HLA-DR are depicted as a function of MOI for GFP^{neg} (bystander; upper panels) and GFP^{pos} (infected; lower panels) cells present in the same MVA-infected culture. Each line represents data from one donor. (D) Production of TNF-α and IP-10 in DCs that were mock-treated, matured with 100 ng/ml LPS, or infected with MVA-GFP at an MOI of 2 was analyzed by flow cytometry at 20 h p.i. Brefeldin A was added during the last 12 h of culture. Results are presented as percentages of cytokine-positive cells in each quadrant from one representative experiment. (E) DCs were infected at increasing MOI, stimulated with 100 ng/ml LPS, or mock treated for 24 h. Then, they were washed, loaded with CEF peptides, and placed in culture with syngeneic PBLs at a 1:25 DC/PBL ratio on ELISPOT plates coated with an antibody against human IFN-γ. The average number of CEF-specific IFN-γ spot-forming cells (SFC)/10^6 PBLs (± SD; $n = 3$) is depicted for each condition. In panels B, D, and E, results for a representative experiment of 3 are shown.

cytokine production. We selected two molecules to analyze, TNF-α and IP-10, as they have recently been described to participate in the interplay between DCs and viral pathogens (35) and were also found in high levels in the supernatants from MVA-infected DCs. For this, TNF-α and IP-10/CXCL10 production in DCs infected with MVA-GFP was evaluated by flow cytometry. The percentages of cells staining positive for these mediators in the infected cultures correlated with the production levels obtained by Luminex technology in the supernatants, where IP-10/CXCL10 levels were higher than those found for TNF-α (Fig. 2). Notably, we found that, while there was a higher percentage of $TNF\text{-}\alpha^+$ cells within the infected GFP^{pos} population, the frequency of $IP\text{-}10^+$ cells was consistently much higher in GFP^{neg} (bystander) DCs (Fig. 4D).

These results suggest that this chemokine could be implicated in the development of the immune response to MVA, as it has been assigned an important role in the immune response to other viruses (31).

Finally, we hypothesized that MVA-infected DCs would be less efficient than bystander DCs in the induction of IFN-γ by T cells, since they expressed lower levels of maturation markers and produced less IP-10/CXCL10, which has been demonstrated to favor T-cell effector functions such as proliferation and antigen-specific IFN-γ secretion (18). In order to test this, we performed an ELISPOT assay in which DCs were infected at increasing MOIs (from 1 to 4) and left to mature for 24 h. After this incubation, cells were washed and viability was assessed by trypan blue exclusion. Viability decreased in inverse relation to the MOI (data not shown), which is consistent with published data regarding the dynamics of apoptosis induction in MVA-infected DCs (10). Then, an equal number of viable cells was pulsed with CEF peptides and put in contact with syngeneic lymphocytes at a 1:100 ratio. We found that the number of CEF-specific IFN-γ-producing cells was highest at the lowest MOI and decreased as MOI increased (Fig. 4E). From these results, it seemed that, as the proportion of infected/bystander DCs increased in the culture, the antigen presentation by MVA-infected DCs was less efficient.

The results depicted in this section demonstrate that, at least in this *in vitro* setting, at 20 h p.i. MVA-infected DCs were still able to produce TNF-α but were not phenotypically or functionally mature. Instead, bystander DCs in the culture (accounting for more than the 70% of the DC culture) were able to mature and were probably responsible for T-cell activation.

Bystander DC maturation occurs in the presence of both supernatant and infected apoptotic cells. We next aimed to characterize the mechanisms by which MVA-induced bystander DC activation occurred. We could envisage that, in an MVA-infected DC culture, soluble mediators produced by infected DCs could achieve maturation of bystander DCs, as reported for other viruses (3, 39). Another possibility was that infected cells themselves would induce maturation of bystander DCs. It has been observed that canarypox (24) and measles (43) virus-infected apoptotic DCs were able to induce maturation of bystander DCs.

To test which of these factors contributed to MVA-induced bystander DC maturation, immature DCs were infected with MVA at an MOI of 5 and incubated for 24 h. This high MOI was used to ensure that over 90% of the cells in the culture were infected. As expected, the proportion of viable cells determined by trypan blue exclusion at 24 h p.i. was usually less than 10% of the initial amount (mean = 8.5%; range = 4.0 to 10.0%; $n = 4$). Annexin V-PI staining revealed that, under these conditions, there was a variable proportion of cells in early (annexin V^+ PI^-) and late (annexin V^+ PI^+) apoptosis for the different donors (data not shown). Then, infected cells and supernatants were harvested and separated by centrifugation at 400 × *g*. Supernatants were filtered with a 0.22-μm syringe filter to eliminate large debris and some of the remaining virus; apoptotic infected cells were washed three times to eliminate virus that might still be attached to the surface. Both preparations were added in different amounts to immature DCs from the same donor. After another 24-hour incubation, CD86 and HLA-DR expression was analyzed by flow cytometry. CD86 expression increased in a dose-dependent manner after stimulation with both supernatant dilutions (Fig. 5A) and apoptotic infected cells (Fig. 5B). In the case of HLA-DR, a dose-dependent increase in expression was clearly observed after incubation with the supernatant from infected cells (Fig. 5C), whereas incubation with MVA-infected apoptotic DCs led to an increase in relative MFI in 3/4 of the donors, but in this case, dose dependency was not always observed (Fig. 5D). For the higher doses, the increases in relative mean fluorescence intensity (rMFI) obtained with these preparations were similar to those obtained after infection at an MOI of 1 (Fig. 1B) (CD86 rMFI mean = 4.7 ± 1.2; HLA-DR rMFI mean = 2.1 ± 0.2). However, the maximal proportion of infected DCs at an MOI of 1 was 26.1% (Fig. 4A), which would be equivalent to a supernatant dilution of 1/8 or an infected-DC/DC ratio of 1:4. The increases in rMFI obtained at these supernatant dilutions or infected-DC/DC ratios were less than the one obtained with direct infection, which suggests that the different factors might act in an additive manner to induce bystander DC maturation.

In order to assess whether DCs matured in the presence of supernatant or MVA-infected apoptotic DCs were functional at inducing IFN-γ production by T cells, mixed cultures were performed as before and IFN-γ production was studied by flow cytometry after 3 days. Consistent with our previous results, DCs matured with either stimulus were able to induce production of IFN-γ both in $CD4^+$ and in $CD8^+$ T cells (Fig. 5E), indicating that in both cases maturation was functional.

To evaluate the possibility that remaining viral particles still present in the preparations could contribute to bystander activation, both supernatants and infected DCs were titrated in BHK cells, and the amounts of virus, calculated as the MOI, were 0.0003 and 0.02 for undiluted supernatants and infected cells at a 3:1 ratio, respectively (data not shown). As we had previously observed that MOIs lower than 1 did not induce significant increases in CD86 and HLA-DR expression (Fig. 1), it would be unlikely that the infective virus contained in these preparations was responsible for bystander maturation. Nevertheless, to reveal if the remaining infective viral particles and other virus components produced during infection could have a stimulatory effect, two additional experiments were performed. In one experiment, DCs were infected at an MOI of 5 as before and the supernatant was obtained after 24 h. Then, the sample was split in two, and one-half of the supernatant was centrifuged at 30,000 × *g* for 1 h at 4°C to eliminate most of the virus it contained and then filtered with a 0.22-μm syringe filter. Elimination of the remaining virus was confirmed by immunostaining. The differently processed supernatants were added to immature DCs, and CD86 relative MFI was analyzed after 24 h. Although the amount of remaining virus was reduced below detection levels by the additional centrifugation step, there was no difference in the degree of maturation achieved by the two preparations (data not shown). To study the effect of the remaining virus on the infected apoptotic cell preparations, DCs were infected at high MOIs (5 and 20). We hypothesized that the percentage of infected cells at these high MOIs would not differ significantly but that the amount of virus associated with these cells would. In fact, analysis of GFP expression at 6 h p.i. revealed 89.0% and 94.3% of infected cells for MOIs of 5 and 20, respectively.

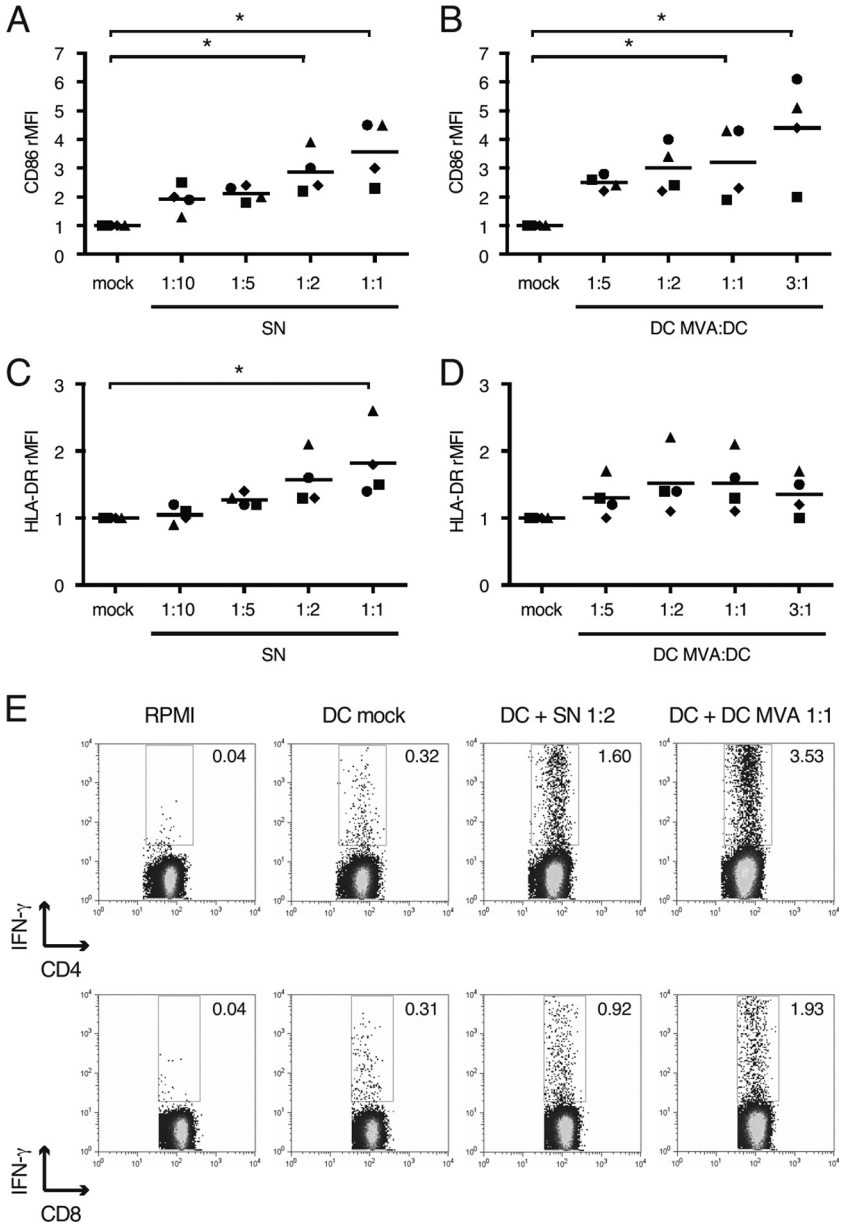

FIG. 5. Activation of bystander DCs can be mimicked by stimulation with supernatant from infected cultures or infected apoptotic cells. DCs were infected at an MOI of 5, washed, and left in culture. After 24 h, supernatants (SN) were harvested and filtered with a 0.22-μm syringe filter while infected cells (DC MVA) were washed repeatedly to eliminate remaining virus. (A to D) To study bystander maturation, both preparations were added in graded doses to autologous DCs. CD86 (for panel A, SN, and for panel C, DC MVA) and HLA-DR (for panel B, SN, and for panel D, DC MVA) expression was analyzed by flow cytometry after another 24 h. Results are shown as rMFIs for each marker, donor, and condition. Lines represent the mean rMFIs for each condition. Statistical difference from the level for mock-infected DCs is shown (*, $P < 0.05$). (E) DCs were incubated with SN at a 1:2 dilution of DC MVA at a 1:1 ratio or complete medium (DC mock) for 24 h, and then they were washed and added to allogeneic PBLs at a 1:20 ratio. PBLs incubated in complete medium were included as negative controls. IFN-γ production was assessed by flow cytometry after 3 days of coculture. Brefeldin A was added for the last 12 h, and then cells were harvested and stained with antibodies against human CD3, CD4, CD8, and IFN-γ. Results are shown as the percentage of IFN-γ^+ cells in gated $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ cells. Results for a representative experiment of 3 are shown.

However, the amount of associated virus measured by immunostaining was 10-fold higher at the higher MOI. In order to inactivate the infective virus, at 24 h p.i. apoptotic cells were washed and UV irradiated. This procedure eliminated infected virus, as assessed by immunostaining. Finally, preparations of apoptotic infected cells at both MOIs, UV irradiated or not, were added to immature DCs from the same donor at a ratio of 1:1, and CD86 expression was assessed by flow cytometry 24 h later. Apoptotic cells infected at an MOI of 20 induced a greater increase in CD86 rMFI than cells infected at the lower MOI (data not shown). These results indicated that the amount of associated infective virus played a role in bystander

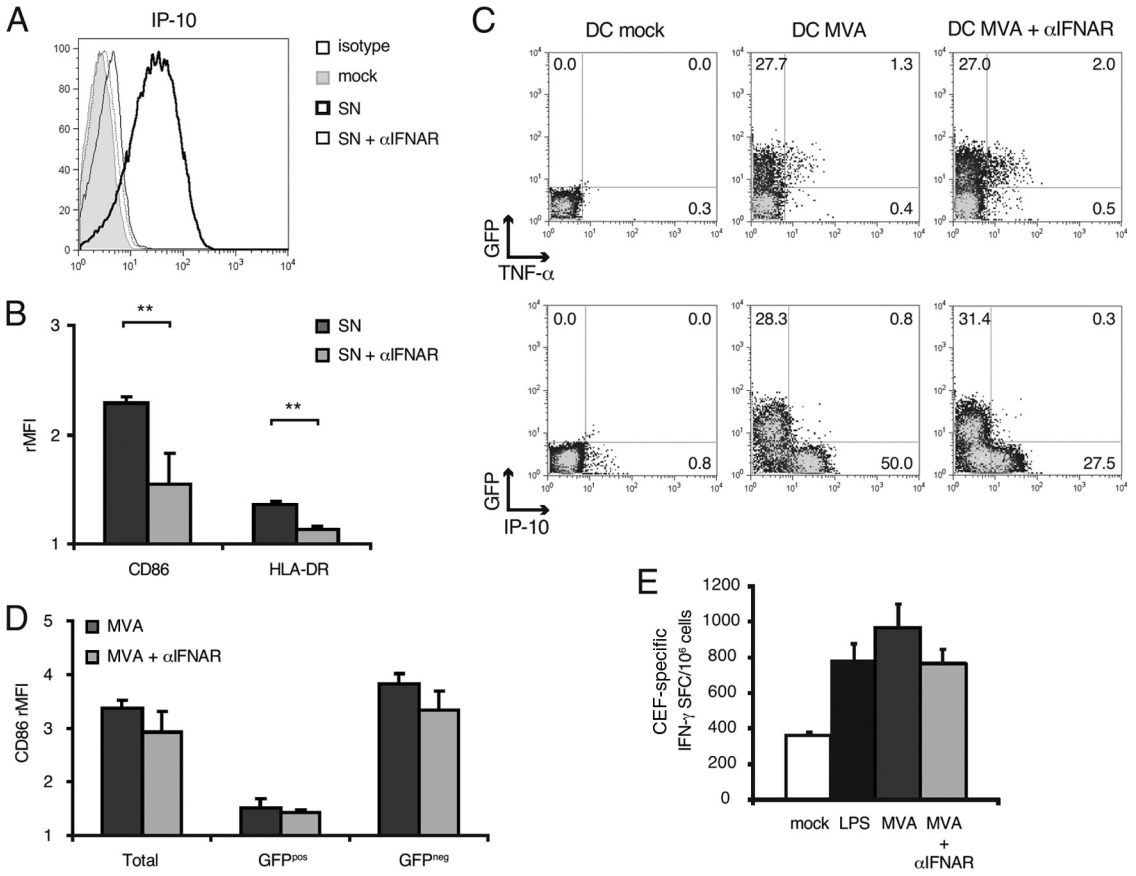

FIG. 6. Type I IFNs contribute to IP-10 production by bystander DCs but do not affect their phenotypic maturation. (A and B) Supernatants (SN) from MVA-infected DCs were prepared as described for Fig. 5. Autologous DCs were pretreated with an antibody against human IFNAR (50 μg/ml) or isotype control antibody for 15 min at 37°C, and then SN was added, leaving the antibody at a final concentration of 10 μg/ml. Incubation with complete medium served as a negative control (mock). (A) IP-10 production was analyzed by flow cytometry after 20 h. (B) Expression of CD86 and HLA-DR was analyzed after 24 h. Results are shown as rMFIs (mean ± SD; $n = 3$) for each condition and marker. **, statistically different from SN ($P < 0.01$). (C to E) DCs were infected with MVA-GFP at an MOI of 1 in the presence of anti-IFNAR antibody (DC MVA + αIFNAR), isotype control (DC MVA), or complete medium (DC mock). (C) TNF-α and IP-10 production was analyzed as described for panel A. (D) CD86 expression was analyzed by flow cytometry after 24 h in the bulk culture (total) or in the GFP^{pos} and GFP^{neg} populations. Results are shown as rMFIs (mean ± SD; $n = 3$) for each treatment. (E) MVA-infected DCs in the presence or absence of anti-IFNAR were incubated for 24 h, washed, loaded with CEF peptides, and placed in culture with syngeneic PBLs at a 1:25 DC/PBL ratio on ELISPOT plates coated with an antibody against human IFN-γ. LPS-matured and mock-treated DCs were included as positive and negative controls, respectively. The average number of CEF-specific IFN-γ-spot forming cells (SFC)/10^6 PBLs (± SD; $n = 3$) is depicted for each condition. In all cases, results for a representative experiment of 3 are shown.

maturation. Moreover, when the virus was UV inactivated, apoptotic cells were no longer capable of inducing bystander DCs maturation, which suggested that either viral transcription in bystander DCs was required or a relevant viral molecule had been destroyed by UV treatment.

In summary, the results presented in this section suggest that maturation of bystander DCs is probably achieved by the combination of soluble factors and virus associated to apoptotic infected cells in the culture.

Type I IFNs contribute to IP-10 production but not to maturation of bystander DCs. Several soluble mediators that are produced by MVA-infected DCs could participate in bystander DC phenotypic and functional maturation. Apart from inflammatory mediators, such as TNF-α, IL-6, or IL-1β, type I IFNs are strong candidates. Padovan et al. have shown that stimulation of human DCs with IFN-α2 led to increased expression of CD80, CD86, and HLA molecules, production of IP-10/CXCL10, and stimulatory capacity of $CD8^+$ T cells in mixed lymphocyte reactions (37). Production of type I IFN by mice DCs after MVA infection has been directly demonstrated (32, 46). In human DCs, upregulation of IFN-α1 mRNA has been reported (23), and we have shown by a Luminex assay a significant increase in the production of IFN-α2 by MVA-infected human DCs (Fig. 2C).

Given this background, we first decided to test whether inhibition of type I IFN in the supernatant from MVA-infected DCs could affect production of IP-10/CXCL10 and maturation of bystander DCs. For this, DCs were preincubated with a blocking antibody against the type I IFN receptor (IFNAR) or an isotype-matched antibody, and then supernatant from infected cells was added. We found that IP-10/CXCL10 production was strongly induced by the supernatant and the anti-IFNAR antibody was able to completely block this effect (Fig. 6A). On the other hand, CD86 and HLA-DR upregulation was

partially blocked by anti-IFNAR (Fig. 6B). These results suggested that type I IFNs produced by MVA-infected DCs were responsible for the induction of IP-10/CXCL10 and that they partially contributed to maturation induced by the supernatant.

We had previously shown that maturation of bystander DCs could be achieved either by the supernatant or by the infected DCs. Therefore, we next examined the contribution of type I IFNs to IP-10/CXCL10 production and maturation in the presence of infected DCs. For this, DCs were infected with MVA-GFP at an MOI of 1 in the presence of the anti-IFNAR or an isotype-matched antibody and then chemokine production was analyzed by flow cytometry on bystander DCs together with TNF-α production on infected cells. As can be seen in Fig. 6C, while TNF-α production on GFP^{pos} cells was unaffected by type I IFN blockade, there was a 50% reduction in the percentage of IP-10/$CXCL10^+$ GFP^{neg} DCs in the culture. We next assessed the levels of CD86 expression and found that this parameter was not affected after anti-IFNAR treatment, either on infected DCs or on bystander DCs (Fig. 6D). Moreover, blockade of IFNAR did not modify the ability of MVA-infected DCs to induce IFN-γ production in CEF-specific $CD8^+$ T cells (Fig. 6E). Together, the results presented in this section indicate that induction of IP-10 production is only partly mediated by type I IFNs and that these mediators do not contribute to phenotypic and functional maturation of bystander DCs after *in vitro* infection with MVA.

DISCUSSION

MVA is a strong and promising candidate in vaccination against a series of cost-burdening high-morbidity diseases, such as malaria, tuberculosis, HIV infection/AIDS, and cancer. At the moment, numerous clinical trials are evaluating the safety and effectiveness of vaccination with MVA in different settings. However, after the failure of the last adenovirus-based HIV/AIDS vaccine clinical trials (11), it has become evident that a greater understanding of the interactions between viral vectors and the immune system is of critical importance in the field of vaccine development. This knowledge is believed to provide clues to help design safer, more-immunogenic, and more-effective vaccines. In the present work, we studied the phenotypic and functional changes induced by MVA infection on DCs and dwelled into the mechanisms leading to DC maturation. Even when MVA has been studied as a vaccine itself or a vaccine vector for more than 30 years, its interaction with DCs has not been fully explored and many questions remain as to how MVA-exposed DCs initiate the adaptive response against the virus. In DCs, MVA-induced block in cellular protein synthesis has been reported to occur as early as 4 h p.i. and apoptosis seems to be evident as early as 12 to 16 h after infection, at a rate even greater than that observed for VV infection (10, 23). These observations would suggest that pathogenic mechanisms induced by MVA are likely to compromise the ability of DCs to mature and induce an immune response. This has been shown to be the case for VV (19). However, MVA has been demonstrated to induce maturation of DCs. This was first evidenced by Drillien et al. (17) in human DCs and then by Liu et al. (32) in murine bone marrow-derived DCs. Maturation of human DCs by an MVA-based HIV vaccine was also very recently reported (8). The results presented in this work confirm and extend these previous publications by demonstrating that MVA induces increases in costimulatory molecules CD83 and CD86 and in HLA-DR expression, although these effects were observed within a narrow window of MOIs of 1 to 3 PFU/cell, coinciding with the recent results observed for murine DCs (36). It is nearly impossible to do an accurate calculation of the possible MOI at which the DC populations would be infected *in vivo*; however, one may speculate that probably low MOIs will be implicated. To this respect, in a recent published paper in which a multigenic recombinant MVA (MVA-CMDR) was employed in a phase I immunogenicity study (14), the authors performed a dose escalation, route comparison trial. The routes analyzed were the intramuscular (i.m.) and intradermal (i.d.), and the MVA-CMDR or placebo was administered i.m. (10^7 or 10^8 PFU) or i.d. (10^6 or 10^7 PFU) at months 0, 1, and 3. The results showed a clear correlation between the dose applied and the immunogenicity found, indicating that cell-mediated immune responses were dose and route dependent, with 10^8 PFU i.m. being the most immunogenic treatment. Thus, if we assume that a higher vaccine dose implies a higher MOI of the DCs implicated during the priming, these results would be in concordance with our *in vitro* findings in the sense that at lower MOIs (<1), no DC maturation was detected.

We also found after MVA DC infection the production of a large array of chemokines and cytokines related to inflammation (MIP1-α/CCL3, RANTES/CCL5, IL-8/CXCL8, IL-6, TNF-α, and others) and T-cell recruitment or activation (IL-12p70, IFN-α2, MIG/CXCL9, and IP-10/CXCL10). Of note, a previous observation by Guerra et al. (23) reported the upregulation of IP-10/CXCL10 mRNA in human DCs. Additionally, Lehmann et al. (28) showed that, in contrast to infection with several other VV strains, MVA induced the expression of MCP-1/CCL2, MIP1-α/CCL3, MIP-1β/CCL4, and IP-10/CXCL10 in the human monocytic cell line THP-1 as well as in primary human monocytes. These authors also demonstrated that MVA, and not other replicative VV strains, consistently triggered the expression of a panel of chemokines in the murine lung, which correlated considerably with the immigration of leukocytes.

The next question to address was whether these phenotypically mature DCs would be functional at activating T cells. Drillien et al. (17) reported that MVA-infected DCs failed to induce IFN-γ production by VV-specific memory T cells in an ELISPOT assay. A similar dysfunctionality was reported by Behboudi et al.; in their work, mouse bone marrow-derived DCs infected *in vitro* with MVA showed an impairment of their T-cell stimulatory capacity in an MLR assay (4). Although this dysfunctionality would be in line with the pathogenic effects of the virus, it comes into conflict with the phenotypic maturation evidenced by others and us. For this reason, we set out to assess DC functionality by different approaches. In mixed cultures, MVA-infected DCs were found to induce proliferation of PBLs and to prime PBLs toward a Th1 cytokine secretion pattern. Additionally, in these same cocultures, MVA-infected DCs were able to activate and induce IFN-γ production in naïve $CD4^+$ and $CD8^+$ T cells. We next evaluated whether the increase in costimulation observed in MVA-infected DCs would be reflected in an increased efficacy to activate specific

T cells. As expected, MVA-infected DCs induced 2 to 3 times more CEF-specific IFN-γ-producing $CD8^+$ T cells than mock-treated DCs. These results support the notion that MVA infection leads to functional maturation of DCs. The level of costimulation expressed on these DCs, together with the cytokine milieu provided by them, seemed sufficient to activate naïve T cells and drive them into a Th1 secretion profile. The differences between previous reports and our present findings may be explained by the different approaches undertaken. In the paper from Behboudi et al. (4), MVA infection of DCs was performed at a high MOI, which might have led to a high percentage of infected DCs in the culture. In this work, we have shown that infected DCs were much less efficient than bystander DCs in activating T cells, which could explain their findings in the MLR assay. On the other hand, Drillien et al. (17) reported that DCs that were directly infected with MVA, despite being mature, could not induce IFN-γ production by autologous VV-specific memory T cells. In this case, the authors assayed a curve of MOIs, so differences cannot be ascribed to the amount of infected cells in the culture. Since we have not formally proven charging of MHC with virally expressed antigen, it could be argued that processing and presentation of antigens could be impaired by MVA. This has been demonstrated for the presentation of antigens by MHC class II molecules after VV infection (30, 40, 48), and Behboudi et al. (4) described downregulation of class I molecules in mouse DCs after infection with MVA. However, we did not observe class I molecule downregulation in human DCs. In addition, the ability of MVA-infected DCs to activate $CD4^+$ and $CD8^+$ T cells *in vitro* in an antigen-specific manner was recently demonstrated by Brandler et al. (8), who described that different antigen-presenting cells, including DCs, were able to induce IFN-γ production by $CD4^+$ and $CD8^+$ HIV-specific T-cell lines after infection with an MVA-based vaccine vector. The results presented by these authors suggest that MVA-infected DCs were able to process and present virally derived antigens to both T-cell populations.

Although MVA has lost many immunoregulatory genes, it is still able to rapidly block cellular protein synthesis and induce apoptosis of the infected cells (10, 26). Moreover, infection of human DCs with MVA at a high MOI blocked LPS-induced maturation (26), indicating that MVA-infected DCs are not fully functional. Thus, we hypothesized that the maturation observed at an MOI of 1 at 24 h p.i. could be mainly occurring in the noninfected bystander DCs in the culture. This hypothesis was supported by the fact that, while most of the cells in the culture upregulated CD86 expression, at most 20% of the cells were GFP^{pos}. Examination of CD86 and HLA-DR levels in GFP^{pos} and GFP^{neg} cells revealed that only bystander cells could upregulate CD86 but that HLA-DR levels were increased in GFP^{pos} DCs from some donors, albeit at lower levels that those found on GFP^{neg} DCs from the same cultures. Class II MHC molecule upregulation in infected DCs could be achieved by fusion of MHC-containing preformed vesicles with the plasma membrane, as has previously been reported (38), while CD86 upregulation would require neosynthesis, a process that would be blocked soon after infection. These results are also supported by the recent work from Norder et al. (36), who reported that there would be a window for the optimal infection of murine bone marrow-derived DCs *in vitro* in order to achieve the maximum level of activation. The authors also underscore that this might explain some of the contrasting results reported in the literature.

At the time point evaluated, not all functions on infected cells were blocked, as we found that GFP^{pos} cells were able to synthesize TNF-α. On the other hand, at 20 h p.i. IP-10/CXCL10 production was restricted to the bystander population of DCs. When we analyzed the ability of MVA-infected DCs to trigger IFN-γ production in CEF-specific cells, we found that as the proportion of infected cells increased, the number of IFN-γ-producing cells decreased, suggesting that infected cells were less efficient than bystander cells at inducing IFN-γ synthesis in antigen-specific cells. These results point to a division of labors between infected and bystander cells in response to MVA, where infected cells would not be able to mature (or if they do, this would occur in a limited time window) but would pass the information to bystander DCs, which in turn would be responsible for T-cell activation. Activation of bystander DCs has been reported for other microorganisms. In some cases, maturation of infected DCs was observed, accompanied by maturation of bystander DCs (29, 39). In other cases, similar to what was found in this work, pathogenic mechanisms impaired maturation of the infected DCs and, therefore, bystander DC activation was proposed as an alternative route for the induction of T-cell responses (9, 35).

Maturation of bystander DCs could be achieved by soluble mediators produced by infected DCs, as was reported for HCMV and HSV-1 (3, 39), or by direct contact with infected apoptotic cells, as MVA infection of immature DCs has been shown to lead to early apoptosis (10). Of note, canarypox (24) and measles (43) virus-infected apoptotic DCs or MVA-infected HeLa cells were able to induce maturation of bystander DCs (8, 17). When we assessed the contribution of these different factors, we found that both supernatant and MVA-infected apoptotic cells were able to induce phenotypic and functional maturation of bystander DCs, albeit with less efficiency than that observed in the direct infection, suggesting that they could be working in an additive fashion. We found several soluble factors produced by DCs after MVA infection that have previously been reported to induce maturation. Proinflammatory cytokines, such as IL-6, TNF-α, and IL-1β, would be strong candidates; additionally, type I IFNs have been reported to contribute to DC maturation in some viral infections (39, 41). There is a considerable amount of evidence that indicates that DCs produce type I IFNs in response to MVA infection (8, 23, 46), and we were able to detect IFN-α2 production. We found that inhibition of type I IFNs before stimulation with the supernatants from infected cells almost completely prevented IP-10/CXCL10 induction but that maturation was partially blocked. These results indicated that type I IFNs could be participating in a paracrine signaling loop to prepare bystander DCs for T-cell activation. However, when we assessed the contribution of these mediators in the presence of the virus, we found only a 50% reduction in IP-10/$CXCL10^+$ GFP^{neg} cells and no decrease in maturation or CEF-specific IFN-γ-inducing ability of MVA-infected DCs after treatment with anti-IFNAR antibody. In other viral infections, such as Rous sarcoma virus (RSV) or herpes simplex virus 1 (HSV-1), maturation of DCs by type I IFN seems to play a predominant role, probably due to the absence or low

levels of proinflammatory mediators (39, 41). However, our results suggest that, during MVA infection, the presence of other mediators makes the contribution of type I IFN to bystander maturation less important. Comparison of the complete blockade of IP-10/CXCL10 production when supernatant was employed as a stimulus with its partial reduction after infection indicates that, in addition to type I IFN, cell-associated factors contribute to IP-10/CXCL10 induction. Several pathways may induce the production of IP-10/CXCL10, such as Toll-like receptors (TLRs) (2, 25) or cytoplasmic receptors (7). Pathogen-associated molecular patterns present in the virus, signaling through TLR-2/TLR-6 or MDA-5 (15, 50), could participate in this process.

In mice, conventional $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ DCs, which human monocyte-derived DCs resemble the most, are located in tissues where, after contact with microorganisms and danger signals, these DCs mature and migrate to lymph nodes, where they can present antigen directly to T cells. In lymphoid tissue, they also come in contact with $CD11c^+$ $CD8\alpha^+$ DCs. The latter population of DCs has been proposed to be more effective in cross-priming (6). There is currently a controversy as to the contribution of direct presentation and cross-presentation in the induction of an immune response to VV. However, Gasteiger et al. (20) have shown in an *in vivo* mouse model that cytotoxic T lymphocyte (CTL) responses against MVA-encoded antigens were dominated by cross-priming. Strikingly, they observed that *in vivo*-infected (GFP^{pos}) DCs expressed smaller amounts of CD80 and CD86 than uninfected (GFP^{neg}) DCs. Based on these observations, the authors speculated that the primary CTL response induced by MVA vaccination did not depend on antigen presentation by directly infected DC but rather is induced by DC that acquire antigen from other infected cells and cross-present it to naïve T cells. The results presented in this study support the idea that a similar scenario might be taking place in MVA-vaccinated human subjects. Further research with human subjects is needed to prove this assertion, but as several clinical protocols already apply MVA as vector vaccines, this should be feasible. This knowledge would provide clues to increase vaccine efficacy for MVA-based vectors, both for infectious diseases and for cancer.

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