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Canarypox and fowlpox viruses as recombinant vaccine vectors: A biological and immunological comparison

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ABSTRACT

Canarypox and fowlpox viruses represent alternative vaccine vectors due to their natural host-range restriction to avian species. Although they cannot replicate in mammals, they correctly express transgenes in human cells and elicit a complete immune response in vaccinated subjects. Several studies have evaluated their genomic differences and protective efficacy in preclinical trials, but detailed information is not available for their transgene expression, cytokine modulation and abortive replication in mammals. This study demonstrates that the heterologous HIV *gag/pol* and *env* genes are more efficiently expressed by fowlpox in non-immune and immune cells. The production of retrovirus-like particles, the longer transgene expression, and a balanced cytokine induction may confer to fowlpox-based recombinants the ability to elicit a better immune response.

1. Introduction

Much effort has been devoted to the development of preventive vaccines using different viral vectors, especially against human immunodeficiency virus (HIV). One of the most used in the late 1980s was the vaccinia virus (VV), which proved effective and highly immunogenic in animal models, but raised safety concerns for skin and central nervous system involvement and for its potential spread to unvaccinated subjects (Picard et al., 1991). Due to their natural host-range restriction to avian species (Baxby and Paoletti, 1992; Somogyi et al., 1993; Taylor and Paoletti, 1988) and their efficient expression of foreign genes also in human cells (Skinner et al., 2005), the canarypox virus (CP) and fowlpox virus (FP) might represent alternative and safer carriers.

Despite the shorter FP genome, molecular data show that CP and FP share high amino-acid identity, significant gene-sequence rearrangements, deletions and insertions (Tulman et al., 2004). They both express cellular gene homologues with immunomodulatory functions, which might be responsible for their different virulence and host-range (Tulman et al., 2004), but CP shows a broader tissue tropism in the permissive avian hosts, generally associated with higher mortality rates (Sadavis et al., 1985) than FP. Both CP and FP have been described as unable to replicate and disseminate infection to non-human and human primates (Taylor et al., 1988b). In particular, Somogyi et al. (1993) described the incomplete replication cycle of FP in cells of monkey and human origin, and the occasional presence of immature forms and mature intracellular virus in infected cells, although the infectivity of this new FP progeny was not determined. Similarly, CP and CP-based recombinants (ALVAC, US Patent 5,766,598) were found to be replication-defective in mammals (Baxby and Paoletti, 1992) and were thus favourably considered to immunise different animal species (Tartaglia et al., 1993; Taylor et al., 1991, 1992), since the host-restricted phenotype of the vector grants naturally attenuated characteristics to the vaccine (Cadoz et al., 1992). Being devoid of safety issues, both CP and FP transcribe and translate the transgenes, resulting in the presentation of foreign proteins to the immune system (Baxby and Paoletti, 1992; Kim and Tripathy, 2001; Taylor et al., 1988a, 1992; Taylor and Paoletti, 1988; Wild

Abbreviations: CP, canarypox virus(es); FP, fowlpox virus(es); CPwt, canarypox wild-type; FPwt, fowlpox wild-type; CP*gp*, CP*gag/pol* recombinant; CP*env*, CP*env* recombinant; CP*gpe*, CP*gag/pol/env* recombinant; FP*gp*, FP*gag/pol* recombinant; FP*env*, FP*env* recombinant; HIV, Human Immunodeficiency Virus; SIV, Simian Immunodeficiency Virus; MVA, Modified Vaccinia Virus Ankara; NYVAC, New York vaccine; VLP, retrovirus-like particle(s); MV, poxvirus mature virions; CEF, chick embryo fibroblast(s); PBMC, peripheral blood mononuclear cell(s); Mϕ, macrophage(s); DC, dendritic cell(s); TEM, transmission electron microscopy.

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et al., 1990) and in the induction of specific humoral and cellular immune responses (Franchini et al., 2004; Taylor et al., 1988b). Moreover, as they do not immunologically cross-react with VV, they can be administered to previously VV-experienced individuals, thus circumventing neutralisation by vector-generated immunity (Irvine et al., 1997). CP and FP recombinants have been used for therapeutic immunisation in macaques during and after discontinuation of antiretroviral therapy (Kinloch-de Leos et al., 2005; Radaelli et al., 2003; Jin et al., 2002), where they were able to restore T-cell responses, prevent viral rebound, and potentially control disease progression. At present, only limited data are available on FP, whereas ALVAC, the most extensively studied CP recombinant, was well tolerated in preclinical trials (Berencsi et al., 2001; Fries et al., 1996), and has shown a good safety profile and induction of immune responses (Belshe et al., 2001; Cao et al., 2003; Corey et al., 2001; Goepfert et al., 2005; Gupta et al., 2002; Jin et al., 2002) with only minor adverse reactions (de Bruyn et al., 2004). Conversely, contrasting data have been obtained in humans by candidate CP- and FP-vectored vaccines, where their safety has not always been paralleled by high immunogenicity, as in macaques (Belshe et al., 2001; De Rose et al., 2006; Kelleher et al., 2006; Kresge, 2009). In particular, although the recent RV144 trial based on CP recombinants has shown 31.2% risk reduction of HIV infection (Kresge, 2009), its real global efficacy is still debated for the limited number of infected subjects (Rerks-Ngarm et al., 2009). However, none of these studies have been aimed at comparing the differences in their abortive replication, transgene expression, and cytokine/chemokine modulation (Nacsa et al., 2004).

We previously demonstrated that a different viral morphogenesis takes place in non-permissive Vero and MRC-5 cells infected with either CP or FP, with no production of retrovirus-like particles (VLP) by the CP*gp* recombinant. Surprisingly, a complete replicative cycle was shown after FP-infection of Vero cells, with production of immature and mature poxvirus virions (MV) and VLP only by the FP*gp* recombinant (Pacchioni et al., 2010). The aim of the present study was to determine the absence of infectivity of the new FP progeny produced by non-permissive Vero cells, and to use wild-type and recombinant CP and FP carrying the HIV *gag/pol* and *env* transgenes for the evaluation and comparison of: (i) the expression of RNA transcripts over time in different cell types, including immune peripheral blood mononuclear cells (PBMC) and macrophages (Mϕ); (ii) the transgene protein expression; and (iii) the ability to modulate the differentiation of dendritic cells (DC) and cytokine expression in immune cells.

2. Materials and methods

2.1. Cells

The specific-pathogen-free primary chick embryo fibroblasts (CEF), monkey kidney cells (Vero) and human lung fibroblasts MRC-5 were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated calf serum (Gibco/Invitrogen, Carlsbad, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (P/S). Human PBMC, DC and Mϕ were cultivated in RPMI 1640 medium supplemented with glutamine (Gibco/Invitrogen), 10% heat-inactivated foetal calf serum (Gibco/Invitrogen), and P/S.

2.2. Preparation of human PBMC, Mϕ, and DC

PBMC were purified from the "buffy coat" of healthy donors by centrifugation on Ficoll-Paque plus (GE Healthcare, Uppsala, Sweden), and used the same day. Mϕ were obtained after plating PBMC ($2–4 \times 10^6$ cells/ml), and removing non-adherent cells after 48 h. DC were generated from purified PBMC enriched either for $CD14^+$ monocytes or for $CD3^+$ cells using a magnetism-activated cell-sorting system (Miltenyi Biotec, Auburn, CA), as previously described (Sallusto and Lanzavecchia, 1994). The cells were cultured at 10^6 cells/ml for 6 days in the presence of DMEM containing 500 U/ml IL-4 and 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Peprotech, Rocky Hill, NJ). The immature DC phenotype was ascertained by staining with a FITC-conjugated monoclonal antibody against CD1c (anti-BDCA-1) (Miltenyi Biotec) and analyzed by flow cytometry. Mϕ and DC were used 3 and 6 days after culturing.

2.3. Viruses

The recombinant avipox viruses CP*env* and FP*env*, carrying the complete HIV-1_{SF2} *env* gene were constructed by *in-vitro* homologous recombination (Radaelli and De Giuli Morghen, 1994) between the insertion plasmids carrying the *env* gene inside the flanking sequences of the C5 locus (CNPV028, ankyrin repeat gene) of the attenuated vaccine strain of CPwt (US Patent 5,756,103), and the F7 locus (FPV046, 3-β-hydroxysteroid dehydrogenase/steroid isomerase gene) of the attenuated vaccine strain of FPwt (US Patent 5,766,599). The recombinant avipox viruses CP*gp* (ALVAC-SIV-*gp*) and CP*gpe* (ALVAC-SIV-*gpe*), carrying the *gag/pol* and *gag/pol/env* genes of SIVmac251, obtained through the courtesy of G. Franchini (NIH, NCI, Bethesda, MD), were inserted into the C5 (US Patent 5,756,103) and C3 locus (Pal et al., 2002), respectively. The FP*gp* recombinant, containing the *gag/pol* genes of SIVmac239, was kindly provided by D. Panicali (Therion Biologics Corp., Cambridge, MA). All of the infections were performed at 5 PFU/cell, unless specified otherwise. Trivalent CP*gpe* was used at 5 PFU/cell, while co-infection with FP*gp* and FP*env* was performed at 5 PFU/cell/recombinant. As the SIVmac239 and SIVmac251 *gag/pol* nucleotide sequences have 99% identity (corresponding to 98% *gag* and 99% *pol* amino acid identity), these genes from the two SIV strains are used indifferently as immunogens. The CP and FP *env* gene expression was driven by the vaccinia virus H6 promoter (VVH6), the CP *gag* gene expression by the early/intermediate vaccinia I3L promoter and the FP *gag* gene expression by the 40 K (H6) promoter.

2.4. Ultrastructural analysis by transmission electron microscopy (TEM)

Confluent Vero and CEF were infected with 5 PFU/cell by the CP*gp* and FP*gp* recombinants for 1 h at 37 °C and collected 3 days post infection (p.i.). After centrifugation at $1000 \times g$ for 10 min, the cells were all fixed in 2.5% glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M Na cacodylate buffer, pH 7.4, for 1 h at 4 °C, then rinsed twice, and post-fixed in buffered 1% OsO_4 at 4 °C for 1 h. The specimens were dehydrated through a series of graded ethanol solutions and propylene oxide, and embedded in Poly/Bed 812® epoxy resin mixture. Sectioning was performed with a Sorvall MT2B ultramicrotome equipped with a diamond knife. After staining with water-saturated uranyl acetate and 0.4% lead citrate in 0.1 M NaOH, ultra-thin sections were viewed with a Philips CM10 electron microscope.

2.5. Virus production from restrictive and replication-permissive cells

To determine viral production from replication-restrictive cells, confluent Vero cells were infected with 5 PFU/cell of either CP or FP for 1 h at 37 °C. After three days, 1 ml of the supernatant was used to infect fresh Vero cells, with re-infection repeated three times. The supernatants from the first-to-fourth infection were harvested to reveal the viral presence by PCR amplification, and to

infect replication-permissive CEF. Cells were scored for plaques for 6 days, and the presence of poxviral sequences was determined by PCR amplification. For DNA extraction, 150 μl of Vero and CEF supernatants were lysed with 20% SDS in TNE buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) in the presence of 200 μg/ml proteinase K. DNA was also extracted from cell lysates of Vero and CEF at the fourth passage of reinfection and from mock-infected Vero cells. After phenol extraction, the DNA was precipitated, resuspended in H_2O, and 100 ng used in a final volume of 20 μl. DNA sequences coding for the CP RNA polymerase gene were amplified using the V259 (5′-TTT-ATT-GCG-AGA-GCG-TGT-TG-3′, nt 294686-294705) and V260 (5′-CCA-CAG-TTT-TCG-CAG-ACG-TA-3′, nt 294973-294992) primers, while the DNA sequences coding for the FP RNA polymerase gene were amplified using the V257 (5′-ATG-CGT-TCT-CGG-GAC-TTC-TA-3′, nt 220916-220895) and V258 (5′-GCC-GCG-ACT-AAG-TCA-TTC-TC-3′, nt 220524-220543) primers. After optimizing the PCR conditions for the different primers, amplifications were carried out in mixtures containing 1 μM of each primer, 200 μM of each dNTP, 2 mM $MgCl_2$, and 0.025 U of Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). For both CP and FP, the PCR conditions were 94 °C for 1 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

2.6. Immunofluorescence

Immunofluorescence was performed on Vero cells infected with either the CP or the FP recombinants at 1 PFU/cell to verify the expression of the *gag/pol* and *env* gene proteins. Briefly, the cells were seeded on sterile glass coverslips at a density of $2 \times 10^5/35\,mm^2$ dish. After an overnight incubation at 37 °C, the cells were rinsed twice with wash buffer (0.2% BSA, 0.1% NaN_3 in phosphate-buffered saline [PBS]) (PBS-BSA). They were then fixed with 2% methanol-free formaldehyde (Polysciences) in Ca^{++}- and Mg^{++}-free PBS (PBS^-) for 10 min at room temperature, followed by 100% cold acetone for 5 min at 4 °C. After washing, the cells were incubated for 1 h at room temperature with a pool of heat-inactivated sera. For the *gag/pol* proteins, 1:50-diluted SIV-positive monkey sera were used, and for the *env* proteins, 1:100-diluted HIV-1-positive human sera. After washing, the samples were incubated in the dark for 1 h at room temperature with 1:50-diluted FITC-conjugated rabbit anti-monkey (Sigma, St Louis, MO, USA) or FITC-conjugated goat anti-human antibodies (Organon Teknika Cappel, Durham, NC). All of the antibody dilutions were performed in PBS-BSA. Mock-infected as well as CPwt- and FPwt-infected Vero cells were used as negative controls. The samples were examined under a Zeiss Axioskop fluorescence microscope and the percentage of fluorescent cells was counted over the total cells in bright field in ten different ocular fields at 400× final magnification.

2.7. Transgene expression in replication-restrictive Vero and MRC-5 cells

Confluent mammalian Vero and MRC-5 cells were infected with the CP*gp*, CP*env*, FP*gp*, FP*env* recombinants, or with their non-engineered counterparts, at 5 PFU/cell for 1 h at 37 °C. The cells were rinsed twice with PBS^-, scraped from the Petri dishes with a rubber policeman every 3 days for 4 weeks, and centrifuged at 1500 × *g* for 5 min. After lysis, the RNAs were extracted using the RNeasy mini kit and treated with RNase-free DNase I (Qiagen, Valencia, CA), as per Manufacturer's instructions, to eliminate any cellular or viral DNA. RT-PCR was performed using the Access RT-PCR system kit (Promega, Madison, WI). Briefly, 100 ng of RNA was used in a final volume of 20 μl in the presence of 1 μM of each primer, 250 μM of each dNTP, 1 U Tfl, 1 U AMV, and 2 mM (*gag/pol* gene) or 1 mM (*env* gene) $MgSO_4$. The *gag/pol* SIVmac239 primers V127 (5′-GAT-AGA-GCC-AGC-ACA-AGA-AGA-3′, nt 3636-3657) and V128 (5′-AGT-TGA-TCT-CTG-CCT-TCT-CTG-3′, nt 4305-4325) were used to obtain a 690-bp DNA fragment and to determine the presence of *gag/pol* transcripts. The HIV-I *env*-specific primers V91 (5′-TAG-GAC-CAG-GGA-GAG-CAT-TT-3′, nt 935–954) and V92 (5′-TCA-TAT-CTC-CTC-CTC-CAG-GT-3′, nt 1401–1420) were used to obtain a 486-bp DNA fragment and to verify the presence of *env* transcripts. Reverse transcriptase reactions were performed at 45 °C for 45 min, followed by 2 min at 94 °C. PCR amplifications were carried out for 35 cycles at 94 °C for 30 s, 59 °C (*gag/pol* gene) or 57 °C (*env* gene) for 30 s, and 68 °C for 45 s. The reactions were followed by a final incubation at 68 °C for 7 min. Human β-actin was seen as a band of 518 bp after amplification of 50 ng RNA in a final volume of 20 μl and the V84 (5′-CTG-ACT-ACC-TCA-TGA-AGA-TCC-T-3′ nt 630–651) and V85 (5′-GCT-GAT-CCA-CAT-CTG-CTG-GAA-3′ nt 1147–1127) primers. RNA amplification for human β-actin was carried out using the conditions described above, except that the annealing temperature was 58 °C, with 1 mM $MgSO_4$. The PCR products were run on 1% agarose gels and the gel images were acquired using a Speedlight Platinum apparatus (Lightools Research, Encinitas, CA). The RT-PCR products were quantified by using the ImageJ software (Rasband, 2009).

2.8. Transgene expression in immune cells

PBMC and Mϕ were infected with 5 PFU/cell of all of the recombinants (CP*gp*, CP*env* or FP*gp*, FP*env*) for 1 h. RNA was extracted as described above, and the *gag* and *env* genes were amplified with the V127/V128 and V91/V92 primers, respectively. FP primers V257/V258 and CP primers V259/V260 were also used to amplify the poxvirus RNA polymerase transcripts and to obtain a 373-bp and a 306-bp DNA fragment, respectively. The RT-PCR products were quantified by using the ImageJ software (Rasband, 2009).

2.9. Determination of CD80 DC differentiation marker by FACS analysis

DC were infected with the wild-type and recombinant CP and FP at 37 °C with 5 PFU/cell for 1 h. One hour before infection, and 6-, 12- and 24-h after infection, the cells were rinsed twice with PBS^-, incubated with FITC- and PE-conjugated monoclonal antibodies or matched isotype controls: anti-BDCA-1(CD1c)-FITC (Miltenyi), anti-CD80(B7-1)-FITC (SouthernBiotech, Birmingham, UK), and anti-CD14-FITC (Caltag Lab., Burlingame, CA). After a 30-min incubation at room temperature, the cells were rinsed with washing buffer (2% foetal calf serum, 0.1% NaN_3 in PBS^-) and analyzed on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San José, CA).

2.10. Cytokine induction in human immune cells

PBMC, Mϕ and DC obtained from a pool of healthy donors were infected with the wild-type and recombinant CP and FP at 37 °C for 1 h. After a 24-h incubation, the supernatants from the infected immune cells were harvested, centrifuged at 1500 × *g* to remove cell debris, and screened for the secretion of Th1-type (IFN-γ, TNF-α, IL-2, IL-12), Th2-type (IL-13, IL-14, IL-15, IL-10), and Th1/Th2-type (GM-CSF) cytokines using the Bio-Plex Pro™ Human Cytokine Assay (Bio-Rad, Hercules, CA), following the Manufacturer's specifications. Briefly, after pre-wetting the wells, the plated cytokine-coated beads were washed in a vacuum apparatus (Millipore, Bedford, MA) before adding 100 μl of each undiluted sample. Premixed standards, serially diluted in cell culture medium, were also used, as well as a negative control. After a 30-min incubation, the samples were washed and treated with 25 μl of detection antibody. The reaction was revealed with 50 μl streptavidin-PE. These experiments were performed in duplicate at room temperature in

the dark, and all of the incubations were carried out on an orbital shaker, at 300 rpm. The fluorescence intensity was determined by a Bio-plex 200 System (Bio-Rad) and cytokine quantification was performed by the Bio-plex Manager 4.0 software. The data are reported as fold-increases *versus* mock-infected cells.

2.11. Statistical analyses

Statistical analyses were performed using one-way ANOVA parametric and non-parametric tests and Bonferroni/Newman-Keuls analysis of variance, using the GraphPad Prism software, version 2.0, as well as the Student's *t*-test. The statistical significance was set as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3. Results

3.1. The abortive replication of CPgp and the absence of VLP production in mammalian cells is due to intracellular restriction

We previously demonstrated by ultrastructural analysis that CP replication is more restricted than the FP one in mammalian cells (Pacchioni et al., 2010). Of all the abortive forms of replication (including viroplasm, viral factories, crescents, poxvirus immature and mature virions) only viroplasm was present after CP infection of Vero and MRC-5 cells, whereas all the replicative forms were observed after FP infection of these same cells.

The expression of *gag/pol* transgenes by CP and FP recombinants can result in VLP production. These pseudovirions are very important as immunizing antigens, but they were detected in mammalian cells only after FP*gp* infection (Pacchioni et al., 2010). Since the transgene espression by CP*gp* and FP*gp* is driven by different promoting sequences, to verify whether the absence of VLP was due to the type of promoter or to cellular restriction, both non-permissive Vero cells and replication-permissive CEF were infected. Although by electron microscopy VLP were not seen in Vero cells after CP*gp* (Fig. 1 A) but only after FP*gp* infection (Fig. 1B), they were produced by CEF after infection with either CP*gp* or FP*gp* recombinants (Fig. 1 C and D). This suggests that the absence of VLP production by CP*gp* depends on cellular restriction, not on the type of promoter.

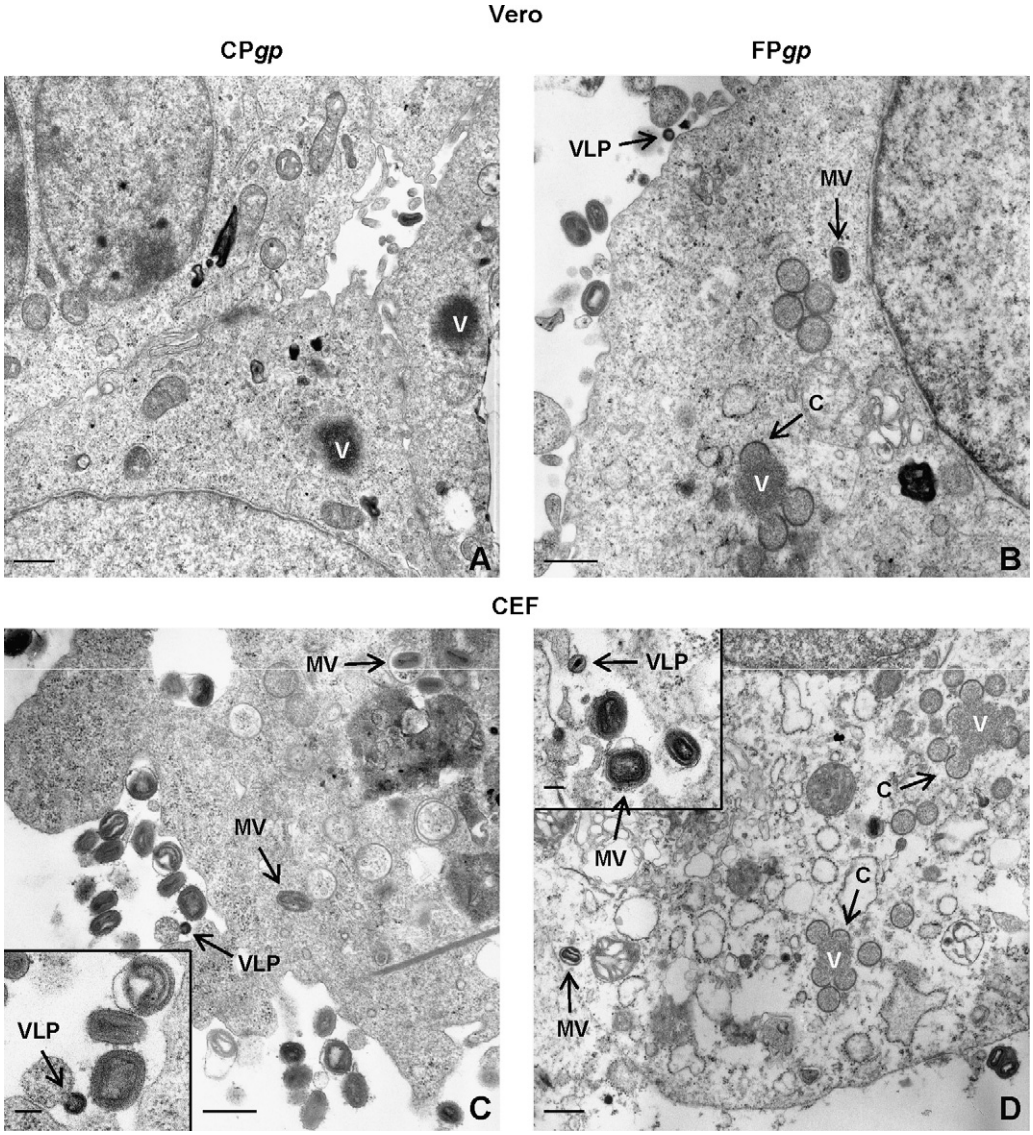

Fig. 1. Ultrastructure of CP*gp* and FP*gp* in replication-restricted Vero and replication-permissive CEF. To verify the production of VLP in Vero and CEF infected by the CP*gp* or FP*gp*, the cells were fixed 3 days p.i., and processed for TEM. In Vero cells, VLP were never seen after CP*gp* (A) but only after FP*gp* infection (B), whereas they were produced by CEF after infection with either CP*gp* or FP*gp* recombinants (C and D), suggesting that the absence of VLP production by CP*gp* depends on cellular restriction. Micrographs show viroplasm (V), crescents (C), poxvirus mature virions (MV), and VLP. Bar: 500 nm. Inset bar: 100 nm.

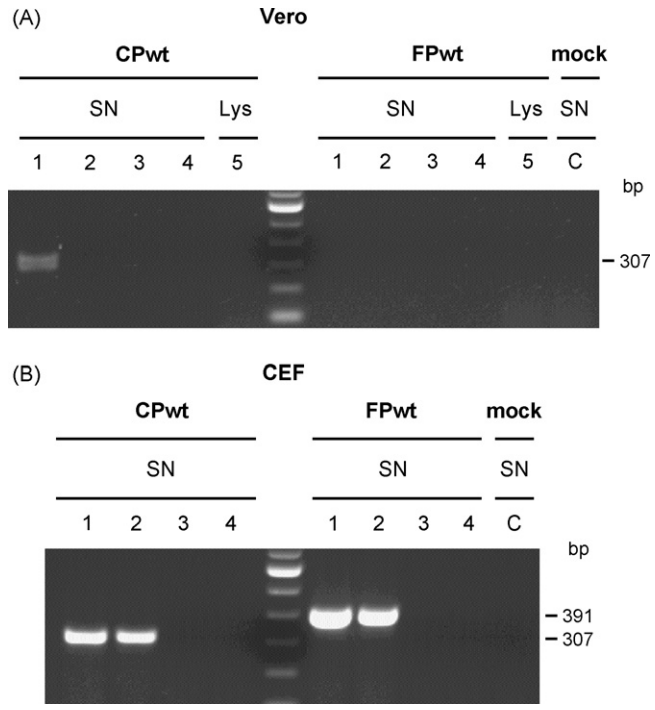

Fig. 2. Viral replication of CPwt and FPwt in non-permissive mammalian cells. Viral DNA could only be amplified (307-bp) from Vero cell supernatants at the first passage after CP infection (A, lane 1), but was undetectable thereafter (A, lanes 2–4) and after FP infection (A, lanes 1–4). Viral DNA could be amplified from CEF infected with supernatants from Vero cells at the first two passages of re-infection (B, 307 and 391 bp, lanes 1–2), but the absence of viral DNA at the 3rd-to-4th passages in CEF (B, lanes 3–4) suggests that only residual inoculum was amplified. Viral DNA was undetectable in cell lysates at the 4th passage of Vero (A, lanes 5) and in the supernatant of mock-infected Vero and CEF cells (A and B, lanes C).

3.2. Vero and MRC-5 cells are replication-restrictive for both CP and FP

CP and FP avipox viruses can be used as safe vaccine vectors because of their intrinsic inability to productively replicate in non-avian cells (Baxby and Paoletti, 1992; Taylor et al., 1988b). However, we have previously observed at ultrastructural level poxvirus mature virions, although only in Vero cells infected with FP, but not with CP, nor after infection of other mammalian cell types (Pacchioni et al., 2010). Therefore, to determine whether this new FP progeny found by electron microscopy was infectious, Vero cells were infected with either CPwt or FPwt and the supernatants were used to re-infect naïve Vero cells. This passage was repeated serially four times and, at each passage, the Vero cell supernatant was harvested and used for viral detection by PCR. The supernatant of each passage was also used to infect replication-permissive CEF to verify the persistence of any residual virus or the release of new infectious viral progeny (Fig. 2). The results showed that viral DNA could be amplified from the supernatant of CP-infected Vero cells only at the first passage, showing a 307-bp band (Fig. 2A, lane 1), but it was always absent thereafter (Fig. 2A, CPwt lanes 2-4) and in the supernatant of FP-infected cells (Fig. 2A, FPwt lanes 1-4). Viral DNA could be amplified from the supernatant of CEF only at the first and second passages of re-infection, where bands of 307 and 391 bp were detected in the CP- and FP-infected CEF, respectively (Fig. 2B, lanes 1-2), thus suggesting the presence of residual virus used for infection, undetectable after the second passage. No viral DNA could be amplified from cell lysates at the fourth passage of reinfection of Vero (Fig. 2A, lanes 5) and from mock-infected Vero and CEF cells (Fig. 2A and B, lanes C).

Table 1
Presence of different replicative forms and VLP in permissive and replication-restrictive cells after CP and FP infection.

		Permissive cells	Non-permissive cells				
		Avian	Simian	Human			
		CEF	Vero	MRC-5	PBMC	Macrophage	DC
VLP	CPgp	++	–	–	–	–	–
	FPgp	+++	+++++	+	+	+	+
V	CP	–	+	+	–	–	–
	FP	–	+	+	–	–	–
MV	CP	++	–	–	–	–	–
	FP	+++	+++	–	–	–	–

The different forms described in CP- and FP-infected cells have been calculated as the average number/20 cells/48 field images. 20 is the average number of cells/section. Data are expressed as follows: –, 0; +, 1–100; ++, 101–200; +++, 201–300; ++++, 301–400; +++++, >400. VLP, retrovirus-like particles; V, viroplasm; MV, poxvirus mature virions.

3.3. FPgp expresses the transgene at higher levels in Vero cells than CPgp

We previously demonstrated (Pacchioni et al., 2010) that VLP are present in different cell types only after infection with FP*gp* recombinants, but absent in the same cells after CP*gp* infection (Table 1), in spite of the ability of this recombinant to produce VLP in replication-permissive CEF (Fig. 1). It was therefore important to determine the level of *gag/pol* transcripts after infection by CP*gp* and FP*gp* in non-permissive mammalian cells. After RNA isolation from infected Vero and MRC-5 cells every three days p.i. for 27 days, transgene transcripts were detected as a 690-bp DNA fragment up to day 9 p.i. after CP*gp* infection and up to day 18 after FP*gp*. A higher expression, as determined by densitometric analysis, was seen after FP*gp* infection in Vero cells (Fig. 3A), whereas only minimal differences were present between CP*gp* and FP*gp* in MRC-5 cells (Fig. 3B). The amplification of human β-actin RNA (518 bp) over time is shown.

3.4. FPenv expresses the transgene longer and at higher levels than CPenv both in Vero and MRC-5 cells

The expression of the *env* transgene after infection by the CP*env* and FP*env* recombinants was also tested. The mRNA isolated from infected Vero and MRC-5 cells showed that the gene carried by the FP*env*, amplified as a band of 468 bp, was expressed earlier (Fig. 4A, right *vs.* left) and for a longer time (Fig. 4A and B, right *vs.* left). In particular, the FP transgene expression extended up to day 27 p.i., whereas the CP transgene expression extended up to day 21 and 18 p.i. in Vero and MRC-5 cells, respectively (Fig. 4A and B). The expression levels, as determined by densitometric analysis, were higher in MRC-5 than in Vero cells (Fig. 4B *vs.* A) after both CP*env* and FP*env* infection. The similar amplification of human β-actin RNA (518 bp) over time confirmed the differences in the *env* expression of the two recombinants.

3.5. FP recombinants express the gag/pol and env proteins in a higher number of Vero cells than CP recombinants

To determine whether RNA transcripts were followed by synthesis of the corresponding viral proteins, and hence to verify and compare the level of expression of the *gag/pol* and *env* transgenes, immunofluorescence was performed on infected Vero cells. The percentage of fluorescent cells was higher after infection with FP*gp* (Fig. 5, 2b, 71%) and FP*env* (Fig. 5, 3b, 77%) than after infection with CP*gp* (Fig. 5, 2a, 36%) and CP*env* (Fig. 5, 3a, 42%). No fluorescence was seen in the negative controls (Fig. 5, 1a and 1b).

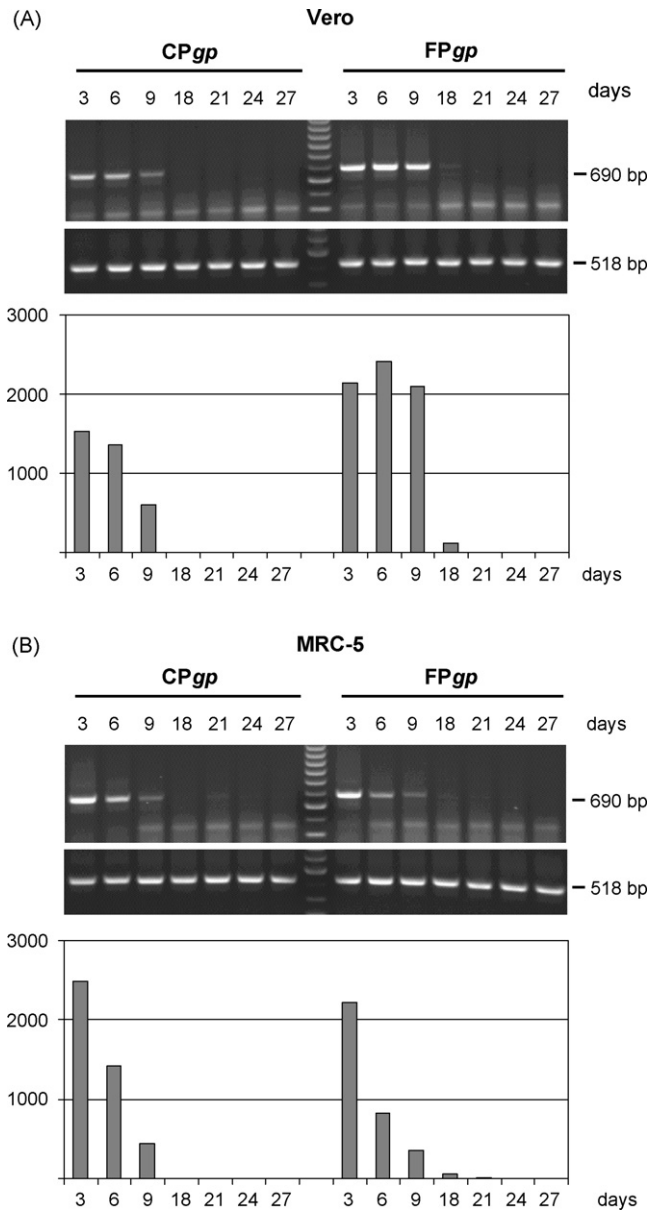

Fig. 3. Expression of the *gag/pol* transcripts over time by the CP and FP recombinants in replication-restrictive mammalian cells. The expression of *gag/pol* was evaluated by RT-PCR for 27 days after infection of Vero (A) and MRC-5 (B) cells with CP*gp* and FP*gp*. Infected Vero and MRC-5 cells show *gag/pol* 690-bp transcripts up to day 9 p.i. after CP*gp* infection and up to day 18 after FP*gp*. A higher expression, as determined by densitometric analysis, was seen in FP- than CP-infected Vero cells (3A), whereas only minimal differences were found in MRC-5 cells after CP or FP infection (3B). Amplification of human β-actin RNA (518 bp) is shown. MW; 100-bp ladder.

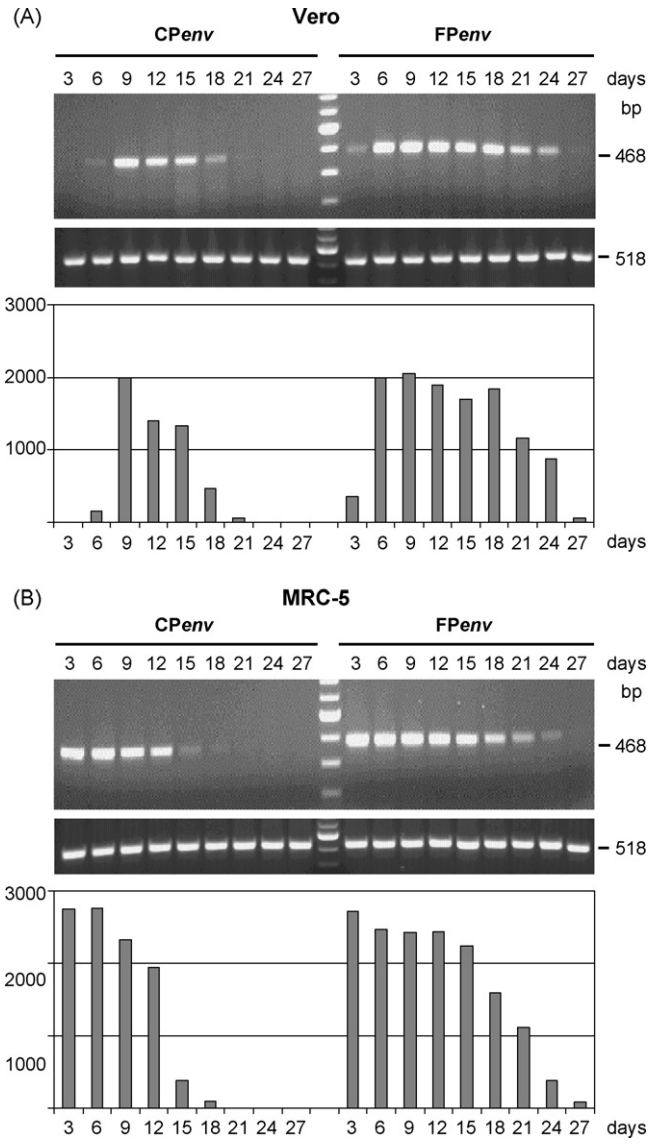

Fig. 4. Expression of the *env* transcripts over time by the CP and FP recombinants in restrictive mammalian cells. After infecting Vero and MRC-5 cells with CP*env* and FP*env*, the expression of the transgene was evaluated by RT-PCR every 3 days for 27 days. The expression levels, as determined by densitometric analysis, extended up to day 27 p.i. for FP*env* transcripts (468-bp), and up to days 21 and 18 p.i. in Vero and MRC-5 cells (A and B) for CP*env*. The expression was always higher in MRC-5 than in Vero cells (4B *vs.* A). Overall, FP*env* showed longer and greater levels of the transgene. Amplification of human β-actin RNA (518 bp) is shown. MW; 100-bp ladder.

3.6. Env expression is significantly higher in FP- than CP-infected immune cells

The different transcription levels of the *gag/pol* and *env* transgenes by the CP and FP recombinants in Vero and MRC-5 cells prompted us to determine whether infection and transgene expression also occurred in immune cells, which are pivotal for the immune response. After mRNA extraction from human PBMC and Mϕ infected with the CP and FP recombinants, the amplified *gag/pol* and *env* transcripts were quantified and compared (Fig. 6). Overall, the *gag/pol* expression was much lower than the *env* expression, and only showed a significant increase at 12 h p.i. after infecting PBMC with FP*gp* (12 h *vs.* 1 h p.i. $p < 0.001$; ANOVA parametric test). Conversely, the *env* transgene expression was always increasing with time in cells infected with FP*env*, and decreasing with CP*env*. In particular, the *env* transgene expression both in Mϕ and PBMC underwent a decrease after infection with CP*env* (12 *vs.* 1 h p.i., $p < 0.01$) and an increase after infection with FP*env* at 12 and 24 h p.i. (12-24 h *vs.* 1 h p.i., $p < 0.001$) also when compared to their CP*env* counterpart ($p < 0.001$).

The amplified *gag/pol* and *env* transcripts were also compared with the CP and FP vector-coded RNA polymerase to verify whether the transgene transcription may depend on the specific CP and FP RNA polymerase activity. The experiments were performed twice with similar results and showed that the expression of *gag/pol* and *env* generally correlated with the CP and FP viral RNA polymerase levels.

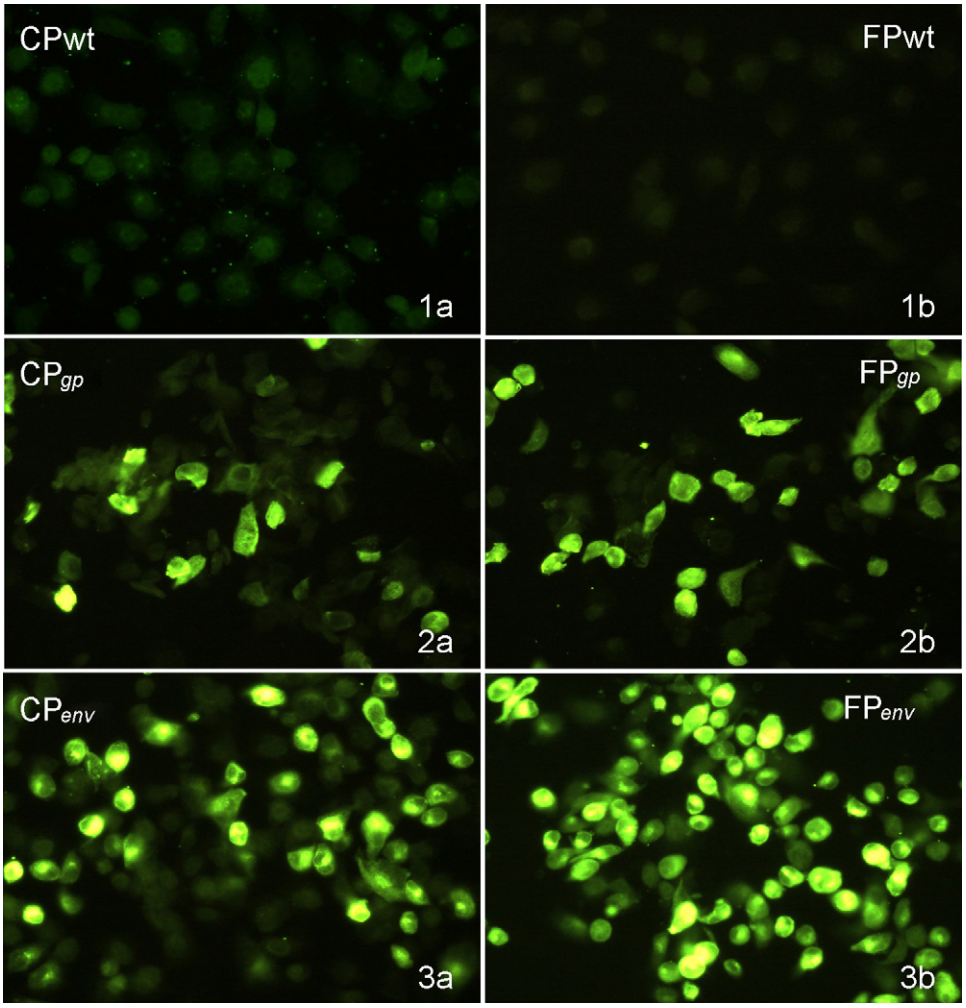

Fig. 5. Heterologous proteins expression by immunofluorescence in replication-restrictive cells. Immunofluorescence of infected Vero cells was performed to compare the expression of the *gag/pol* and *env* proteins. The percentage of fluorescent cells was counted over the total cells observed in bright field in ten different ocular fields at 400× final magnification. A higher percentage of positive cells was found after FP*gp* (2b, 71%) and FP*env* (3b, 77%) than after CP*gp* (2a, 36%) and CP*env* (3a, 42%) infection. No immunofluorescence was detected in CPwt and FPwt negative controls (1a, b).

3.7. CD80 is differently expressed in DC by CP and FP

CD80 was evaluated as a DC differentiation marker to determine whether the *gag/pol* and *env* transgenes carried by the CP and FP vectors can modulate DC maturation (Fig. 7), which may allow a more efficient antigen presentation and elicit a better immune response. Overall, a progressive decrease of CD80 expression was present at 6 and 12 h p.i. compared to their respective previous time point, which was significant at 12 h p.i. for CP*gpe*- ($p < 0.01$, ANOVA non-parametric test) and FPwt-infected cells ($p < 0.05$). However, this decrease was followed by an increase of CD80 at 24 h *vs.* 12 h p.i. for CPwt, CP*gp*, CP*gpe* ($p < 0.001$) and CP*env* ($p < 0.05$) as well as for FP*gpe* ($p < 0.01$). A significant increase was also present after FP*gp*- and FP*gpe*-infection *vs.* FPwt ($p < 0.001$) or *vs.* FP*env* ($p < 0.001$ and $p < 0.01$, respectively) at 24 h. CD80 expression values were reported as the differences between the percentages of CD80-positive virus-infected *vs.* mock-infected cells.

3.8. CytokineTh1/Th2 polarisation is differently modulated by CP and FP

The cytokines expression was evaluated in PBMC, Mϕ and DC after infection with the CP and FP recombinants (Fig. 8). For the analysis, the cytokines were grouped on the basis of the Th1-, Th2- and Th0-type response and the results are described only when statistically significant for the comparison between CP *vs.* FP in the different cell types, and Th1 *vs.* Th2. In PBMC, a Th2 *vs.* Th1 increase was seen after all of the FP infections (Fig. 8A2, $p < 0.05$, Student t-test). In Mϕ, an increase in the Th1 *vs.* Th2 response was seen after both CP and FP infection, and in particular in CPwt, CP*gp*, FPwt and FP*env* (Fig. 8B1, B2, $p < 0.05$). In DC, the Th1 *vs.* Th2 response increased after infection with both CPwt and all of the CP recombinants (Fig. 8C1, $p < 0.001$), and after FP*gp* infection (Fig. 8C2, $p < 0.05$). The CP Th1 response in DC was also higher than the FP one (Fig. 8C1 *vs.* C2, CPwt $p < 0.01$, CP*env* $p < 0.05$, CP*gp* $p < 0.05$).

Overall, the Th1 response was higher in DC after CP infection, whereas the Th2 response was higher in PBMC after FP infection. The data are presented as fold-increases of cytokines in virus-infected *versus* mock-infected cells. No significant differences in cytokine modulation were seen when the recombinants were compared to their wild-type counterparts, or when the transgenes were expressed by the different CP and FP recombinants.

4. Discussion

Several studies with poxvirus-based vaccines have demonstrated the efficacy or failure of different immunisation regimens (Earl et al., 2002; Fries et al., 1996). Due to their natural host-

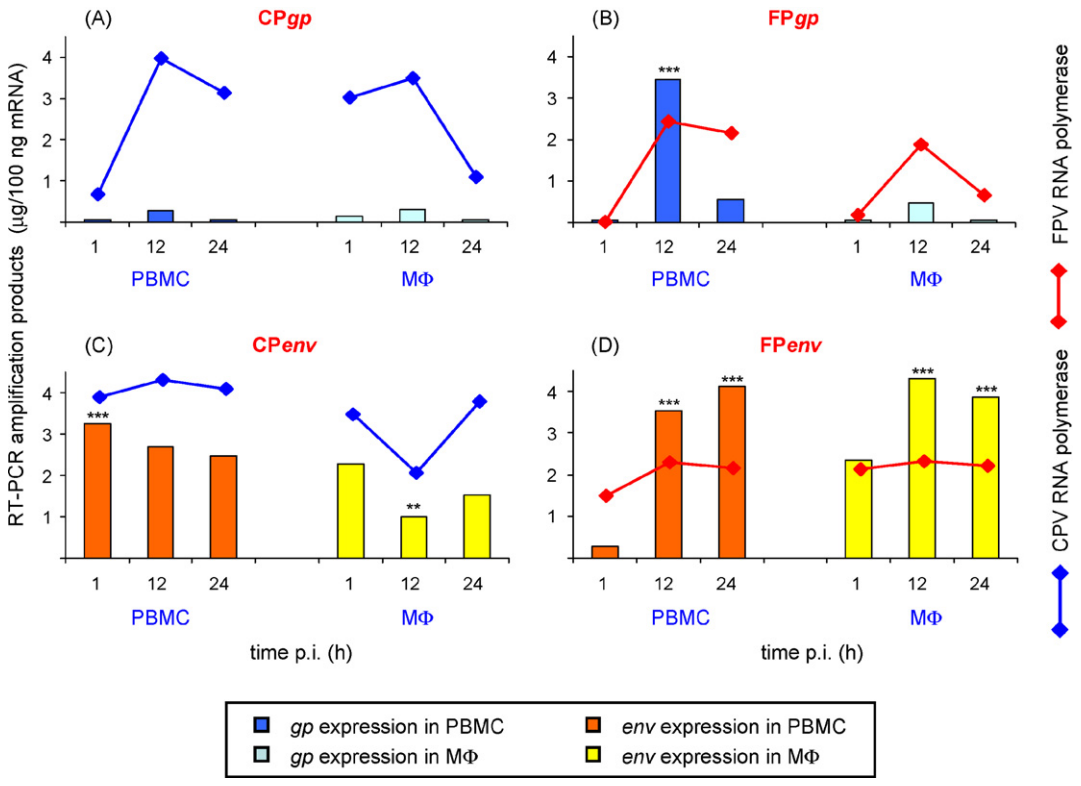

Fig. 6. Heterologous gene transcription in human immune cells. RNA was extracted from PBMC and MΦ infected with the CP and FP recombinants. *Gag/pol* and *env* RNA transcripts were quantified, as well as the CP and FP vector-coded RNA polymerase. The *gag/pol* expression was much lower than *env* expression, which showed a significant decrease in CP MΦ at 12 h and increase in FP MΦ and PBMC at 12 and 24 h. The experiments were performed twice with similar results and showed that the expression of *gag/pol* and *env* generally correlated with the vector RNA polymerase levels. Bars indicate *gp* and *env* transgene expression; lines indicate vector-coded RNA polymerase at different times. Statistical significances using the ANOVA parametric test are shown: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

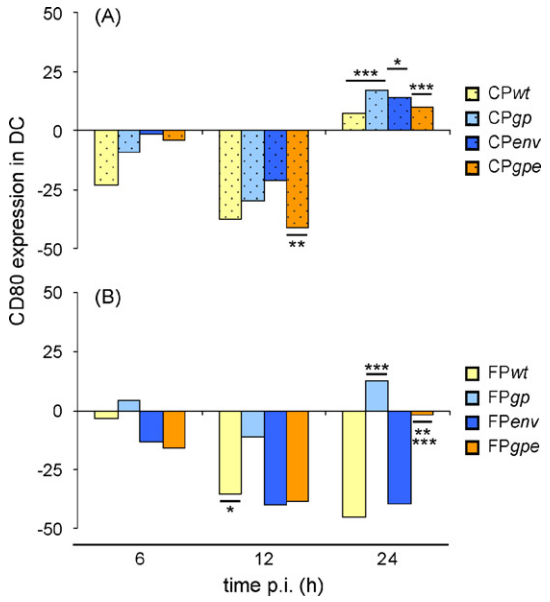

Fig. 7. CD80 expression in human DC. Expression of CD80 as a DC differentiation marker was evaluated by FACS analysis. After infection with the CP and FP recombinants, the cells were incubated for 30 min with an anti-CD80 (B7-1)-FITC monoclonal antibody. A progressive decrease was present in CD80 expression at 6 and 12 h p.i. compared to their respective previous time point, which was significant at 12 h p.i. for CP*gpe*- and FPwt-infected cells. This decrease was followed by an increase at 24 h *vs.* 12 h p.i. for CPwt, CP*gp*, CP*gpe* and CP*env* as well as for FP*gpe*. This increase was also significant for FP*gp* and FP*gpe vs.* FPwt and FP*env* at 24 h. The data were normalized and reported as differences between the percentages of CD80-positive virus-infected and mock-infected cells. Statistical significances using the ANOVA non-parametric test are shown: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

restricted replication to avian species, the CP and FP recombinants have been tested as putative HIV vaccines in preclinical and clinical trials. Although the genomic differences of these two avipox vectors have already been investigated (Tulman et al., 2004), comparisons between the transgene and chemokine expression by their recombinants in different mammalian cells have not been performed. In the present study, we compared the CP and FP recombinants expressing the same HIV-1 transgenes, and we demonstrate that: (i) despite the complete replication and poxvirus mature virions seen by electron microscopy in FP-infected Vero cells, the new progeny is not infectious; (ii) the FP*env* recombinant expresses the transgene for longer and to higher levels in non-immune and immune cells; (iii) the *gag/pol* and *env* proteins are expressed in a higher number of cells by the FP vector; (iv) the FP-induced DC differentiation is dependent on *gag/pol* transgene expression; and (v) the cytokine Th1/Th2 polarisation is differently modulated in immune cells by the two vectors.

To verify the infectivity of the new viral progeny found by electron microscopy in FP-infected Vero cells (Pacchioni et al., 2010), cell supernatants were used to repeatedly infect both Vero cells and CEF. Considering the absence of viral DNA after the second passage in replication-permissive CEF, and the relative heat-stability of poxviruses, which can allow the persistence of infectious virus in the supernatant, the amplified bands should not be ascribed to new infectious viral progeny, but to the initial residual extracellular inoculum still present in culture supernatant of Vero cells. CP amplification from the supernatant of the first round on Vero cells can be explained by virus adhesion in the absence of penetration.

VLP production is the expected product of the expression of the *gag* gene, but their absence after infection of mammalian cells with CP*gp* did not correlate with the similar level of *gag/pol* transcripts expressed by CP*gp* and FP*gp* in Vero and MRC-5 cells. However,

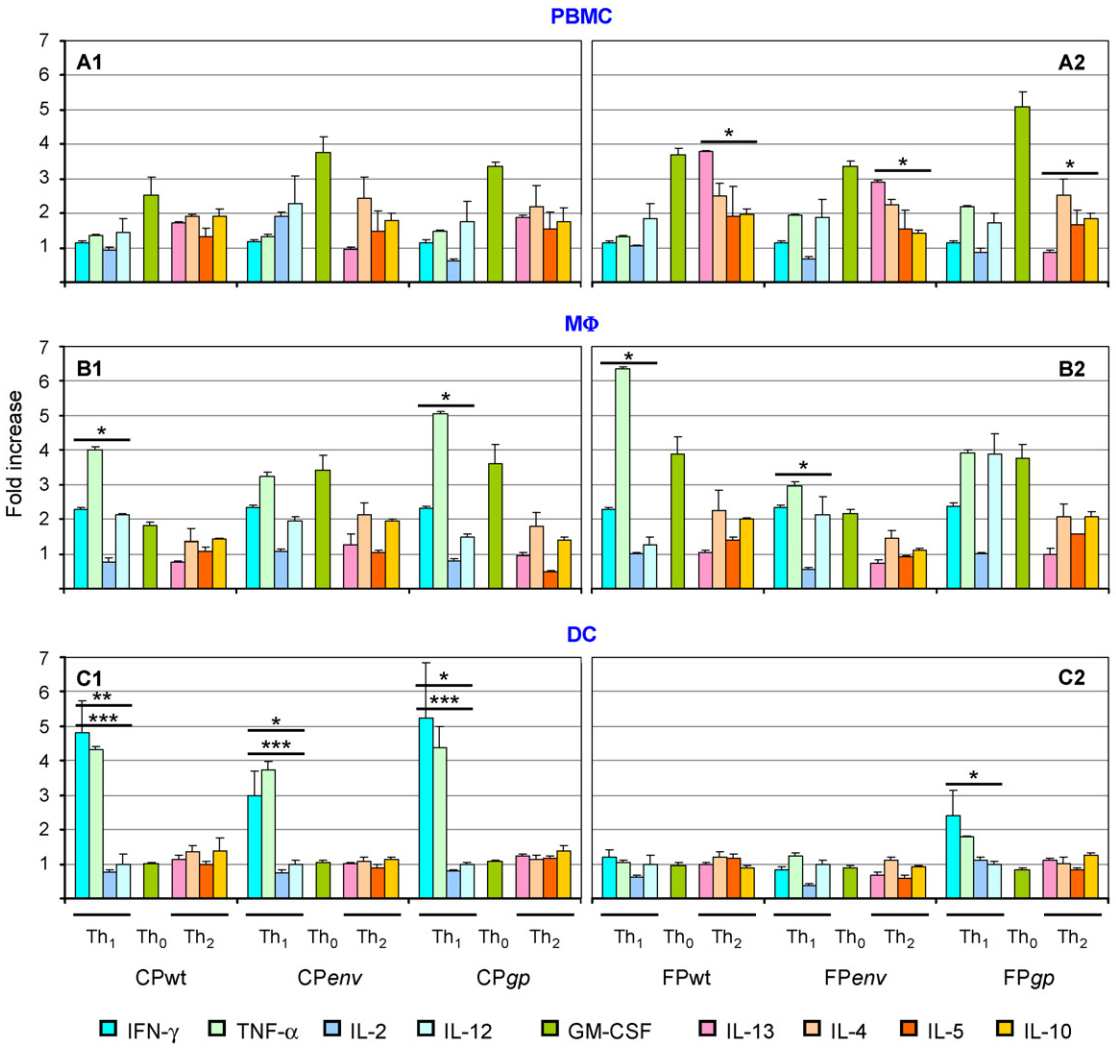

Fig. 8. Modulation of Th1 and Th2 cytokine expression by CP and FP. Cytokine expression was evaluated in PBMC (A), Mϕ (B) and DC (C) obtained from healthy human donors, and infected *in vitro* at 37 °C for 1 h with 5 PFU/cell of the CP or FP wild-type and recombinants. The Th1 response was higher in DC after infection with CP (C1 *vs.* C2), whereas the Th2 response was higher in PBMC after infection with FP (A2 *vs.* A1). The analysis was performed in duplicate using the Bio-Plex Pro™ Human Cytokine Assay. No significant differences were seen between recombinants and their wild-type counterparts. The data are presented as fold-increases *versus* mock-infected cells. Statistical significances using the Student's *t*-test are shown: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the expression of the *env* transgene over time appears higher, earlier and longer lasting in FP- than in CP-infected cells, suggesting that, in spite of the abortive replication of both of the vectors, a better immune response might be obtained using FP. Although not strictly dependent on viral replication, both *gag/pol* and *env* transgene expression appears to correlate with the very restricted CP and more advanced FP morphogenesis shown by electron microscopy in Fig. 1. We suggest therefore that the inability of the CP *gag/pol* proteins to assemble into VLP may also be associated with the more restrictive replication of CP. Surprisingly, the *env* gene expression was higher in MRC-5 than in Vero cells, where replication of both vectors was more restricted. This finding might be explained by a higher poxvirus-induced shut-off of cellular transcription in MRC-5 cells, which may favor a higher expression of the foreign gene. On the other hand, this did not occur when MRC-5 cells were infected with either CP*gp* or FP*gp*. The continuous recruitment of *gag* proteins for the assembly of VLP should also be excluded, as VLP are produced by FP but not by CP. The lower expression of the *gag/pol* than the *env* gene can be explained by the different cellular localisation of the *gag* and *env* products. *Gag* products are in fact synthesized on free polyribosomes, whereas *env* transcripts are attached to membrane-bound polyribosomes, where protein synthesis and glycosylation take place as co-translational events, and thus can persist longer in the cytoplasm. This is also in line with the higher number of cells expressing *env vs. gag* proteins seen by immunofluorescence and the lower expression of the *gag/pol* than the *env* transcripts in PBMC and Mϕ in spite of an adequate level of vector RNA polymerase. The higher number of fluorescent cells might also be ascribed to a more efficient infectivity of FP recombinants.

Although no sign of viral replication was seen by electron microscopy after CP or FP infection of PBMC, as summarized in Table 1, transgene transcripts were always detected by RT-PCR, and at a significantly higher level in FP*env*- than in CP*env*-infected cells. These data appear in agreement with previous analyses showing that transgene expression by FP recombinants persisted for up to 20 days p.i. in immature and mature human DC (Brown et al., 2000). However, no significant correlation was found between the extent of transgene expression and the quality/quantity of Th1/Th2 cytokines.

CP and FP modulate the differentiation process of human DC, resulting in a steep decrease of CD80 soon after infection. However, CP is responsible per se for the restoration of the CD80 marker, with only a limited contribution by the transgenes. Conversely, the

increase of CD80 at 24 h p.i. when using the FP*gp* or FP*gpe* recombinants can only be ascribed to the *gp* transgene expression, not to the vector.

Many studies have suggested that the cytokine profile is important for protection from viral exposure and disease progression (Amara et al., 2002; Hel et al., 2001), and that DC at mucosal sites are the most important cells for immune activation (Steinman, 1991). In this context, the present study indicates that, after enrichment in the Mϕ subset, a significantly higher Th1 *vs.* Th2 response is elicited by both CP and FP, while a different cytokine modulation is induced after infection of PBMC and DC. Although the level of each cytokine in each cell type is often not correlated with the level of the same cytokine in PBMC, due to the interplay of the different subsets of immune cells that always occurs in vivo, it is also important to consider the contribution of each cell subset to the immune response. The Th2-type response in FP-infected PBMC is mainly due to IL-13 and IL-4, and might be a determinant in the ability to elicit humoral immunity. The Th1-type response by CP in DC, independently from the type of transgene, can be ascribed to TNF-α and INF-γ, which, however, might limit its immunogenicity. In fact, although TNF-α expression mediates the recruitment of migratory leukocytes to the site of infection, as TNF-α is associated with apoptosis, its induction might reduce the efficacy of the immune response, all the more that anti-apoptotic genes have not been reported for CP and FP. Moreover, poxviruses express receptor-like proteins that can counteract the virus-induced IFN-γ production, which affects the antigenic presentation of non-replicating poxvirus-based vaccines. In spite of the expected inhibition of IFN-γ in the absence of viral replication, high levels of IFN-γ were present in CP-infected DC. The recently identified product of the FP 016 gene (Puehler et al., 2003), able to neutralize IFN-γ activity, has not been described in CP, suggesting that different anti-IFN-γ strategies are adopted by the two avipox vectors. As we demonstrated by EM in a recent paper (Pacchioni et al., 2010), no sign of vector replication is present in immune cells after infection by avipox viruses.

CP-based vaccines have been extensively studied and have proven to be highly effective when used alone or in prime–boost regimens in macaques (Sabbaj et al., 2000), but they have shown suboptimal activities when applied to humans (De Rose et al., 2006). If the administration of higher doses of a vaccine can increase immunogenicity, the intolerable reactogenic response that can result (Somogyi et al., 1993) makes it advisable to consider a more suitable vector for vaccine construction. The widely divergent level of immunogenicity of avipox recombinants is the result of the interaction of several factors, such as the promoter, the type of transgene, the immunization protocol and the administration route (Cottingham et al., 2006), and cannot be ascribed only to the type or strain of the vector. However, the more advanced replication cycle, the longer transgene expression in human cells, the VLP production by the *gag/pol* transgene, and the more balanced Th1/Th2 cytokine induction might confer to the FP-based recombinant vaccines the ability to elicit a more effective immune response.

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