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Systemically administered DNA and fowlpox recombinants expressing four vaccinia virus genes although immunogenic do not protect mice against the highly pathogenic IHD-J vaccinia strain

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ABSTRACT

The first-generation smallpox vaccine was based on live vaccinia virus (VV) and it successfully eradicated the disease worldwide. Therefore, it was not administered any more after 1980, as smallpox no longer existed as a natural infection. However, emerging threats by terrorist organisations has prompted new programmes for second-generation vaccine development based on attenuated VV strains, which have been shown to cause rare but serious adverse events in immunocompromised patients. Considering the closely related animal poxviruses that might also be used as bioweapons, and the increasing number of unvaccinated young people and AIDS-affected immunocompromised subjects, a safer and more effective smallpox vaccine is still required. New avipoxvirus-based vectors should improve the safety of conventional vaccines, and protect from newly emerging zoonotic orthopoxvirus diseases and from the threat of deliberate release of variola or monkeypox virus in a bioterrorist attack. In this study, DNA and fowlpox recombinants expressing the L1R, A27L, A33R and B5R genes were constructed and evaluated in a pre-clinical trial in mouse, following six prime/boost immunisation regimens, to compare their immunogenicity and protective efficacy against a challenge with the lethal VV IHD-J strain. Although higher numbers of VV-specific IFNγ-producing T lymphocytes were observed in the protected mice, the cytotoxic T-lymphocyte response and the presence of neutralising antibodies did not always correlate with protection. In spite of previous successful results in mice, rabbits and monkeys, where SIV/HIV transgenes were expressed by the fowlpox vector, the immune response elicited by these recombinants was low, and most of the mice were not protected.

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1. Introduction

The original vaccinia virus (VV) smallpox vaccine was administered by scarification. Due to the successful eradication of smallpox worldwide, the use of this vaccine was discontinued from 1980, later to be replaced by VV-derived, second-generation, cell-cultured vaccines, such as ACAM2000® (Weltzin et al., 2003). These vaccines showed similar immune responses as Dryvax®, but were associated with health risks and contraindications (Nalca and Zumbrun, 2010; Wiser et al., 2007), as they could spread to immunocompromised, non-vaccinated subjects (Bray, 2003; Centers for Disease Control and Prevention, 2007; Jacobson et al., 2008; Lane and Goldstein, 2003). In the attempt to develop strains with lower reactogenicity and fewer side effects, and to face the potential re-emergence or accidental/deliberate release of orthopoxviruses (OPXV) in the human population (Cardeti et al., 2011; Megid et al., 2012; Vogel et al., 2012; Whitley, 2003), a third generation of attenuated vaccines was developed. Although not as virulent as variola, the monkeypox (MPXV) and cowpox (CPXV) viruses are also a threat to public health, as they cause mortality

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in underdeveloped countries (Reed et al., 2004) and can become a potential bioweapon if adapted to grow and spread in humans (Lewis-Jones, 2004).

The new attenuated vaccines, which include the VV-derived Lister clone LC16m8 (Kenner et al., 2006) and the most advanced modified vaccinia Ankara (MVA; e.g., the IMVAMUNE® (Kennedy and Greenberg, 2009)), have an improved safety profile and induce more rapid responses (Earl et al., 2004). However, their ability to induce long-term immunity is controversial (Earl et al., 2007; Ferrier-Rembert et al., 2008), and they also fail to induce protective immunity in immunocompromised animals (Edghill-Smith et al., 2005a). MVA also induces lower immunogenicity than the traditional smallpox vaccine (Ferrier-Rembert et al., 2008) and shows a limited replication in mammals (Blanchard et al., 1998). A further caveat may be represented by possible recombination events that may rescue endogenous OPXV genes and generate a fully-replicative genotype (Okeke et al., 2009; Verheust et al., 2012). MVA also failed to protect animals with CD4/CD8 combined immunodeficiency (Wiser et al., 2007) and Rhesus macaques infected with simian immunodeficiency virus showing a very low cell count of the immune repertoire (Edghill-Smith et al., 2005a).

Although the VV antigens that protect against smallpox are not completely defined, neutralising antibodies have mainly been raised against the surface proteins of the two OPXV infectious particles: the mature virions (MVs) released after cell lysis and the extracellular virions (EVs), which are wrapped by an additional envelope (Moss, 2011; Pacchioni et al., 2013; Roberts and Smith, 2008; Smith et al., 2002). In particular, the combined use of the L1 and A27 proteins of MV, and the A33 and B5 proteins of EV has conferred better protection than the individual use of MV or EV proteins (Fogg et al., 2004; Hooper et al., 2002). These subunit vaccines are protective against VV intranasal challenge in mice or MPXV intravenous challenge in monkeys (Buchman et al., 2010; Fogg et al., 2007; Hirao et al., 2011; Hooper et al., 2010).

In the present study, four DNA recombinants that express the VV *L1R, A27L, A33R* and *B5R* genes (called the 4DNAmix) were used, followed by four novel fowlpox (FP) recombinants that express the same genes (Pacchioni et al., 2013) (called the 4FPmix). MVA, which also contains the same genes, was used as a control, either alone or in a prime/boost regimen, followed by the 4FPmix. A direct comparison was performed between humoral and cell-mediated responses and the ability to induce protection in the immunised mice. Attenuated avipox viruses have been developed as novel vectors for the construction of recombinant vaccines against several human infectious diseases (Radaelli et al., 1994; Zanotto et al., 2010). These vectors are restricted for replication to avian species, but permissive for entry and transgene expression in most mammalian cells. They are also immunologically non-cross-reactive with vaccinia (Baxby and Paoletti, 1992), and they can escape neutralisation by vector-generated antibodies in smallpox-vaccine-experienced humans.

2. Materials and methods

2.1. Cells

Specific-pathogen-free primary chick embryo fibroblasts (CEF) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated calf serum (Gibco Life Technologies, Grand Island, NY, USA), 5% Tryptose Phosphate Broth (Difco Laboratories, Detroit, MI, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. Green monkey kidney (Vero) cells, and Balb/C mouse fibroblasts (B77 cells) were grown in DMEM supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin.

2.2. Viruses and fowlpox recombinants

The highly pathogenic IHD-J strain of VV was supplied by S. Dales (University of Western Ontario, London, Canada) (Wilton et al., 1986), and was grown in Vero cells, to use as the challenging virus (1×10^6 PFU/mouse) through the airways. IHD-J was amplified in Vero cells, purified on discontinuous sucrose density gradient, and titrated as described previously (Pacchioni et al., 2013). The 4FP recombinants, FP_{L1R}, FP_{A27L}, FP_{A33R} and FP_{B5R}, that express the L1, A27, A33 and B5 proteins of VV, respectively, were generated in our laboratory by *in-vivo* homologous recombination (Pozzi et al., 2009). Gene insertion was performed downstream of the VV H6 early/late promoter (Rosel et al., 1986), inside the 3-β-hydroxysteroid dehydrogenase 5-delta 4 isomerase gene interrupted by a multiple cloning site. Recombinants were grown and amplified in CEF, and purified on discontinuous sucrose gradients. MVA was kindly obtained by A. Siccardi (Dept. of Biology, University of Milan, Italy), and amplified and purified on CEF, as already described (Soprana et al., 2011).

2.3. Plasmids

The expression plasmids pcDNA3.1$_{A27L}$, pcDNA3$_{L1R}$, pcDNA3$_{A33R}$ and pcDNA3$_{B5R}$ were constructed in our laboratory by insertion of the same genes used for the FP recombinants. In particular, pcDNA3.1 (Invitrogen Corp., San Diego, CA, USA) was used for the *A27L* gene, which was inserted into the HindIII/NotI restriction sites, whereas pcDNA3 (Invitrogen Corp.) was used for the *L1R, A33R* and *B5R* genes, which were separately inserted into the HindIII/XhoI restriction sites. Both pcDNA3.1 and pcDNA3 contain the human CMV promoter. The mixture of these four recombinants is called the 4DNAmix. PcDNA3$_{gag/pol}$ was used as an irrelevant negative control, and described previously (Zanotto et al., 2010).

2.4. Immunisation protocols

Six groups of eight female Balb/C mice (Charles River Laboratories, Wilmington, MA, USA) were immunised by multiple injections. All of the mice were inoculated three times at 3-week intervals, before challenge. After immunisation, all of the mice remained in good health, without loss of weight. Before each inoculation, the mice were anaesthetised with 300 μl 2.5% 2,2,2-tribromoethanol (Avertin®) (Sigma, St Louis, MO, USA) i.p., and bleeding was performed from the retro-orbital eye plexus. The plasma fraction was aliquoted and frozen at −80 °C. Six different prime/boost immunisation protocols were followed (Fig. 1), using: (i) plasmid 4DNAmix (100 μg of each recombinant/mouse; i.m.) in combination with recombinant FP*gagpol* (10^7 PFU/mouse; s.c.) (Group 1); (ii) 4DNAmix (100 μg of each recombinant/mouse; i.m.) in combination with recombinant 4FPmix (10^7 PFU of each recombinant/mouse; s.c.) (Group 2); (iii) recombinant 4FPmix (10^7 PFU of each recombinant/mouse; s.c.) used for the three inoculations (Group 3); (iv) MVA (10^7 PFU/mouse; s.c.) used for the three inoculations (Group 4); (v) MVA (10^7 PFU/mouse; s.c.) in combination with 4FPmix (10^7 PFU of each recombinant/mouse; s.c.) (Group 5); and (vi) pcDNA$_{gagpol}$ plasmid (100 μg/mouse; i.m.) in combination with FP*gagpol* recombinant (10^7 PFU/mouse; s.c.) (Group 6). All of the mice were maintained in accordance with the Italian national guidelines. Mice were observed for signs of disease and weighed daily, provided food and water *ad libitum*. Every effort was made to minimise suffering and, based on predetermined criteria (loss of more than 30% body weight) moribund animals were euthanized. The approval for this study was granted by the ethical Committee of the University of Milan.
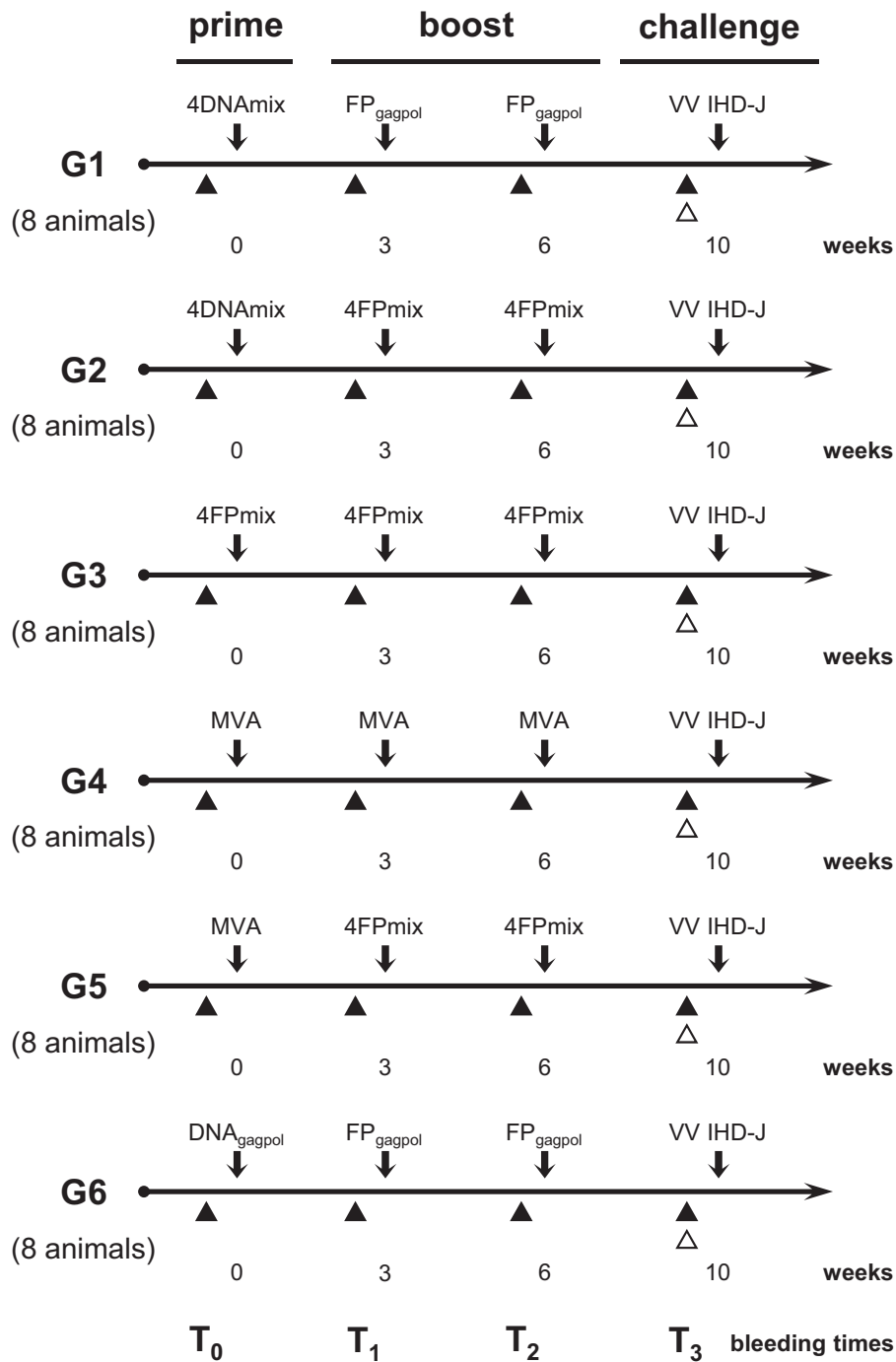
Fig. 1. Immunisation protocols. Six different prime/boost immunisation protocols were followed using 8 mice per group. The *L1R, A27L, A33R* and *B5R* genes were expressed either by pcDNA3 or by FP recombinants, and administered together as the 4DNAmix or 4FPmix, respectively. Each plasmid was administered i.m. at 100 μg/mouse, and each virus was administered s.c. at 10^7 PFU/mouse. MVA was used as a positive control or for priming. Plasmid pcDNA*gagpol* and FP*gagpol* were used as negative controls. G1 to G6, immunisation group numbers.

2.5. ELISA

The mice plasma was tested for the presence of antibodies against the L1, A27, A33 and B5 VV-specific proteins before the first immunisation and after each immunisation. Purified IHD-J virus was plated at a concentration of $2\,\text{PFU} \times 10^5$/well in a 96-well MaxiSorp microtitre plate (Nunc, Naperville, IL, USA) in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4 °C. The ELISA was performed in triplicate, essentially as described previously (Radaelli et al., 2010). The sera were diluted 1:1000 and the reactions were revealed using a 1:1000 dilution of goat anti-mouse horseradish–peroxidase-conjugated sera (DakoCytomation, Glostrup, Denmark) and tetramethylbenzidine substrate (Sigma). Preimmune mouse sera were used as negative controls. The absorbance of each well was read at 490 nm using a 550 microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Virus neutralisation assays

The neutralising activity of the mice sera, that were obtained at different times post-immunisation and after the challenge, was tested in duplicate by measuring the extent of inhibition of

IHD-J infectivity. The assays were performed by preincubation for 1 h at 37 °C in a 48-well plate of an equal volume of IHD-J with heat-inactivated mouse serum, used at different dilutions in DMEM. The viral inoculum was adjusted to give approximately 5×10^2 PFU of IHD-J/ml. Infection was performed in duplicate on Vero cells, and was allowed to proceed for 1 h at 37 °C. The same amount of virus incubated with DMEM was used as a negative control, whereas the virus incubated with hyperimmune mouse serum was used as a positive control. Three days later, 1.5% neutral red was added, and the plaques were counted the next day, as described previously (Pacchioni et al., 2013). Sera from each group at the different times post-infection (p.i.) were pooled before use. Sera from Group 5 were also tested individually. Neutralisation was expressed as the percentage of inhibition of the infection compared to the negative control, where the virus was incubated with DMEM only.

2.7. In-vivo cytotoxicity assays

B77 syngeneic cells were separately infected with each FP recombinant (6 PFU/cell) for 1 h at 37 °C. Five hours later, the cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS⁻), trypsinised, and harvested in DMEM with 5% calf serum, and counted, to prepare a mixture with equivalent numbers of cells infected with each recombinant. The same number of cells infected with FPwt was also prepared. The two cell populations were loaded with two different concentrations of the vital fluorescent carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) dye (Life Technologies) and mixed at a 1:1 ratio (6×10^6 cells infected with the recombinants, as cytotoxic T-lymphocyte (CTL) targets, and 6×10^6 cells infected with FPwt, as a control, for each mouse). Briefly, the cells were incubated separately with 0.75 μM or 0.075 μM CFDA-SE in DMEM plus 1% foetal bovine serum for 30 min, and washed twice with DMEM plus 10% foetal bovine serum before use. The staining and the cell ratio was further checked by flow cytometry and set as a control. Each mouse was injected with 1.2×10^7 cells, in the caudal vein. Two mice for each group of immunised animals were used for this assay, which was performed after the third immunisation, at 10 weeks p.i. Target and control cells were recovered from the spleen 24 h later.

2.8. Ex-vivo cytokine production

Popliteal lymph nodes from 2 mice per each group were dissected mechanically, until single-cell suspensions were obtained. The cells from draining and non-draining lymph nodes were cultured for 5 h at 37 °C with 10 μg/ml brefeldin A (Sigma), 50 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 100 ng/ml ionomycin (Sigma) in complete DMEM medium, and processed for surface and intracellular cytokine staining as described previously (Radaelli et al., 2003). Briefly, after treatment with 2 mM EDTA in PBS and an incubation at 37 °C for 5 min, the cells were washed twice with PBS, surface-stained with a fluorescein-isothiocyanate-tagged anti-CD4 antibody (5 μg/ml), washed again, and fixed and permeabilised with Cytofix/Cytoperm (BD Immunocytometry Systems, San José, CA, USA) for 15 min on ice in the dark. Following two further washes, the cells were stained with anti-IFNγ-APC (14 μg/ml) (BioLegend, BD) and anti-IL4-PE (14 μg/ml) (BD Immunocytometry Systems), incubated for 30 min at 4 °C, washed extensively three times with PBS containing 5% foetal bovine serum, and analysed by four-colour flow cytometry (FACSCalibur, BD).

2.9. Mouse challenge

Preliminary tests were first performed by challenging naïve animals, to evaluate the minimum lethal dose to kill 50% of
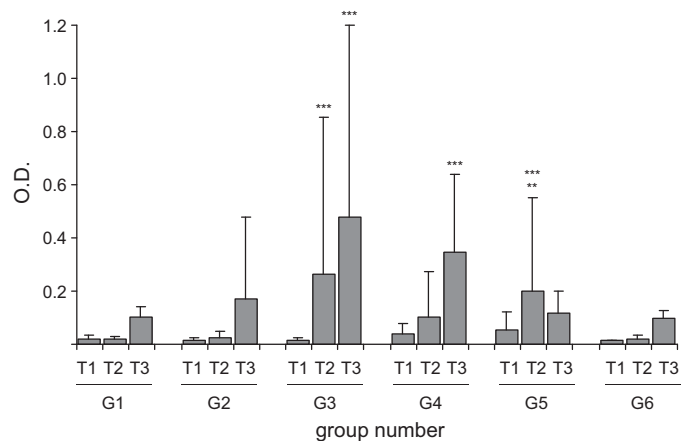
Fig. 2. Analysis of the specific humoral responses. The anti-L1, A27, A33 and B5 antibody levels were determined by ELISA using purified IHD-J VV as the plate-bound antigen. The bars indicate the average of the values of the animals of each group. The responses were compared among the groups, and the mice of G3, G4, and G5 showed significant increases in specific antibody titres at T2 and T3. Statistical differences using one-way ANOVA parametric tests and Bonferroni/Newman-Keuls analysis of variance are shown: (**) $p < 0.01$; (***) $p < 0.001$.

the mice (LD_{50}). These mice were inoculated with high (5×10^5, 20×10^5, 30×10^5 PFU IHD-J), or low (1×10^5, 2×10^5 PFU IHD-J) doses of the VV IHD-J strain, with the LD_{50} defined as 2×10^5 PFU IHD-J. For the experimental assay, six mice out of the eight per group were challenged 6 weeks after the third immunisation, using intranasal administration of 1×10^6 PFU IHD-J in 30 μl PBS⁻, administered through a plastic pipette tip after anaesthetising the animals with 300 μl avertin i.p. All of the mice were followed daily, with measurements of their weight and monitoring for disease symptoms.
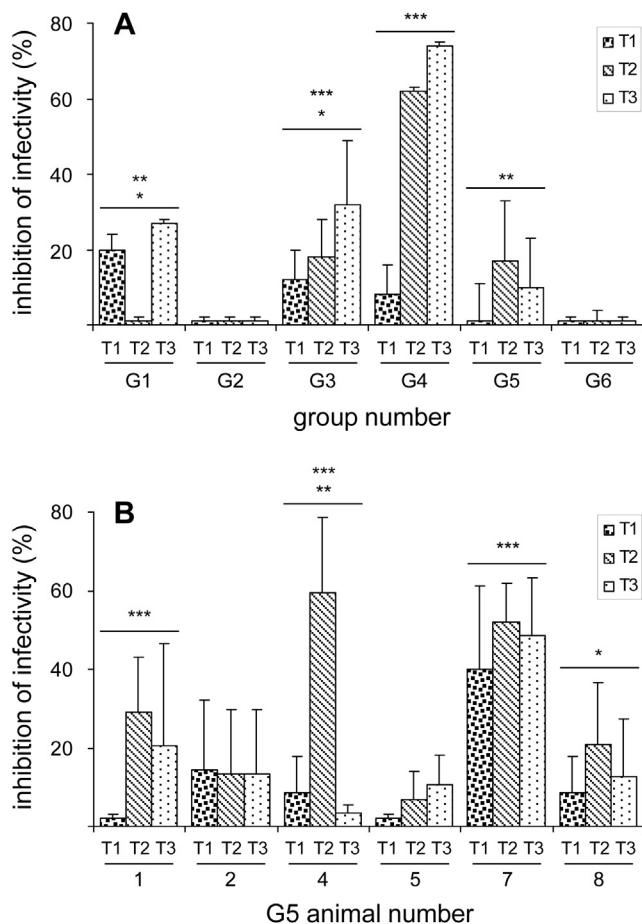
2.10. Statistical analyses

Statistical analyses were performed using one-way ANOVA 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

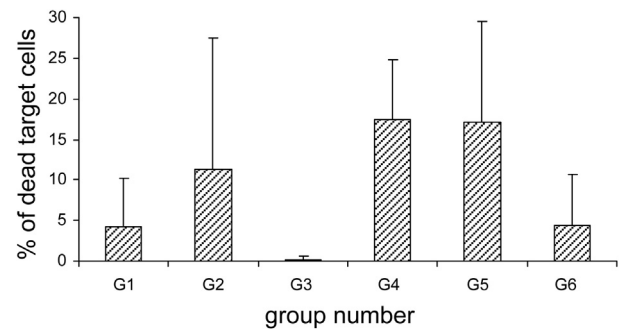### 3.1. Low humoral response in immunised mice

With the aim of developing a vaccine strategy that can protect from OPXV infections, six different immunisation protocols were compared for the raising of antibodies and for effector-T-cell-specific responses in the mice. Both DNA and FP virus recombinants were constructed to prime six groups of eight mice, and they were followed by two boosts with FP recombinants as shown in Fig. 1. All of the animals remained in good health after all of the rounds of immunisation. The specific humoral response against L1, A27, A33 and B5 proteins expressed by DNA and/or FP recombinants was measured in the plasma by ELISA, using the plate-bound purified IHD-J virus as the antigen (Fig. 2). The animals of Group 5 showed a significant increase at T2 ($p < 0.01$ and < 0.001 vs Group 4 and 6, respectively), which was observed in the mice of Group 3 both at T2 ($p < 0.001$ vs Groups 4 and 6), and at T3 ($p < 0.001$ vs Groups 4, 5, and 6). In Group 4, the increase was seen at T3 ($p < 0.001$ vs Groups 5 and 6). The results are shown for the 1:1000 serum dilutions.

Fig. 4. *In-vivo* cell-mediated cytotoxicity. The mice were injected with B77 syngeneic cells infected with the 4FPmix or FPwt and loaded with two concentrations of CFDA-SE dye. The CTL responses were evaluated using flow cytometry, after recovering target and control cells *ex-vivo* from the spleen 24 h after the inoculation. The percentages of dead cells were higher than the negative control in Groups 2, 4 and 5 (G2, G4, G5), with Group 4 and Group 5 being almost similar, although no statistical differences were found.

Fig. 3. Neutralising antibodies elicited by the different mouse immunisation protocols. Virus neutralisation assays were performed on Vero cells after each immunisation. Plaque reduction was quantified as compared to the control, in which the viral inoculum was incubated with the preimmune serum, and expressed as percentages of inhibition of infectivity. (A) Except for Group 2 (G2), all of the groups of mice raised neutralising antibodies against IHD-J, and inhibition of infectivity generally increased after the second immunisation. (B) Sera from mice 1, 4, 7 and 8 of Group 5 showed significantly higher inhibition of infectivity, although not all of these mice were protected. Conversely, mouse 5 of Group 5 was protected in the absence of significant levels of neutralising antibodies. Statistical significances using the Student *t*-test are shown: (**) $p < 0.01$; (***) $p < 0.001$.

number 7 had a neutralising activity >40% ($p < 0.001$ *vs* mice 1, 2, 4, 5, 8), whereas three mice had neutralising activities >20%: mouse 1 ($p < 0.001$ *vs* mouse 5), mouse 4 ($p < 0.01$ *vs* mouse 1 and $p < 0.001$ *vs* mice 2, 5, 8), and mouse 8 ($p < 0.05$ *vs* mouse 5). Conversely, after each immunisation, mice 2 and 5 showed low neutralising activities ≤15%.

3.3. Comparable cell-mediated cytotoxicity is elicited by MVA vs MVA/FP-recombinant vaccines

The antigen-specific CTL activity was analysed *in vivo* on splenocytes. B77 syngeneic cells were infected with each FP recombinant or with FPwt, stained with two different CFDA-SE concentrations, and injected i.v. into the immunised mice. These were evaluated the next day using flow cytometry. Antigen-specific CTL-mediated killing was calculated by the decrease in the ratio between the percentage of B77 cells infected with FP recombinants and B77 cells infected with FPwt, after infusion in the immunised mice. The results are expressed as the percentages of specific killing (Fig. 4), and they show that there were more dead target cells in Groups 2, 4 and 5 than in the negative control, although no statistical differences were found between Groups 2, 4 and 5.
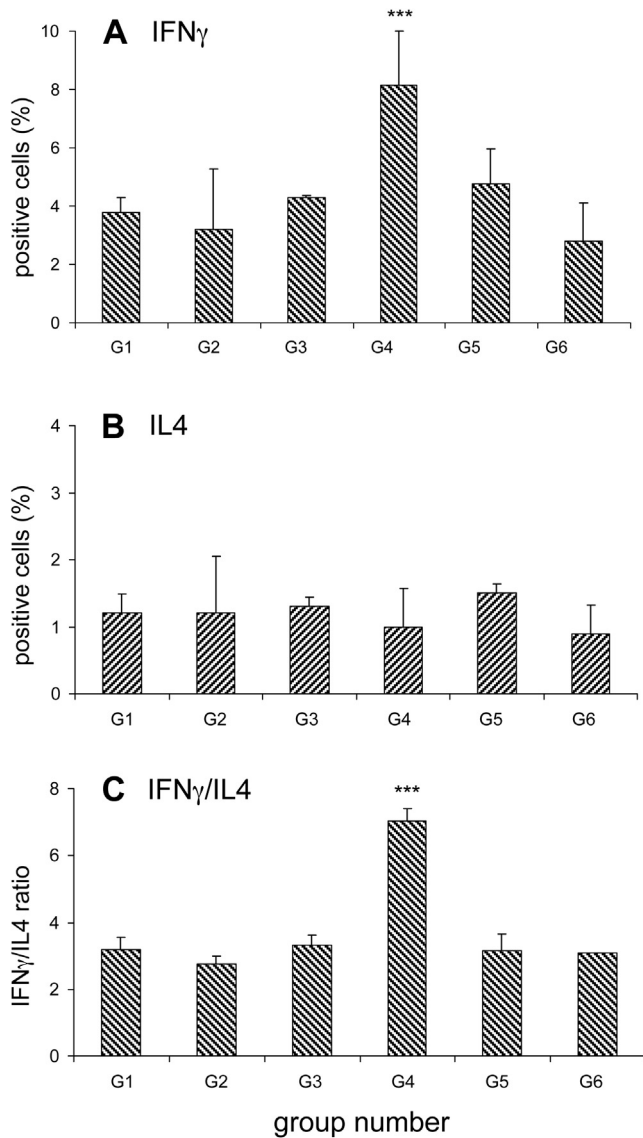
3.4. IFNγ- and IL4-mediated immune responses after vaccination

The secretion of effector cytokines by CD4+ T cells in the mice immunised following the different immunisation regimens was assessed by intracellular cytokine staining. Single-cell suspensions from draining lymph nodes near the site of injection were cultured *in vitro* in the presence of phorbol 12-myristate 13-acetate and ionomycin, and the percentages of CD4+ cells producing IFNγ or IL4, as parameters of Th1 and Th2 responses, were determined by cytofluorimetric analysis (Fig. 5).

No increases in the percentages of cells that produced IFNγ or IL4 effector cytokines were observed in the different mouse groups (Fig. 5A and B), except for Group 4, which showed a significant increase ($p < 0.001$) in IFNγ production *vs* the Group 6 negative control.

3.5. Protection of immunised mice

To evaluate the efficacy of the vaccine-induced responses according to the different immunisation regimens, the mice were monitored for weight and survival after the challenge with the VV IHD-J strain. Preliminary assays showed that mice injected with

3.2. Neutralising activity against IDH-J does not always correlate with mouse survival

To determine whether the humoral responses seen after these regimens of immunisation prevented IDH-J infectivity, neutralisation tests were performed (Fig. 3A). As a whole, a low neutralising activity was raised in Group 1 ($p < 0.01$ *vs* Group 2 and Group 6, Student *t*-test) and in Group 3 ($p < 0.05$ *vs* Group 5, and $p < 0.001$ *vs* Group 2 and Group 6), whereas no neutralising activity was detected in Group 2 or 6, with low neutralising activity in Group 5 ($p < 0.01$ *vs* Group 2 and Group 6). In contrast, the mice of Group 4, which were used as a positive control, raised high levels of neutralising antibodies against IDH-J ($p < 0.001$ *vs* Groups 1, 2, 3, 5, 6). As 50% of the mice of Group 5 (mice numbers 4, 5, 7) were protected from IDH-J infection (see Fig. 6C), the sera from this group were also separately analysed at post-immunisation times, to try to evaluate the correlation between viral neutralisation and survival and the contribution of the specific immunogen (Fig. 3B). The analysis was not performed for animals 3 and 6, that were used for the *in-vivo* cytotoxicity assays. The results were expressed as the average of two determinations, and they showed that mouse

Fig. 5. Functional virus-specific T-cell responses induced by the vaccinations. IFNγ and IL4 production were measured after *in-vitro* stimulation with phorbol 12-myristate 13-acetate and ionomycin in an intracellular staining assay. IFNγ (A) and IL4 (B) were evaluated as parameters representative of Th1 and Th2 responses, respectively. There was a higher number of CD4+ T cells producing IFNγ in Group 4 mice (A, G4), as compared to the other groups of mice, which gave a significantly greater IFNγ/IL4 ratio *vs* the negative control (C). IL4 production was low in all of the groups. Statistical significance using one-way ANOVA parametric tests and Bonferroni/Newman-Keuls analysis of variance is shown: (***) $p < 0.001$.

Fig. 6. Efficacy of the vaccine-induced responses by the immunisation regimens. Different viral doses of IHD-J were tested on naïve mice to define the LD_{50} (A). Six weeks after the third immunisation, six animals per group were challenged by intranasal administration with 1×10^6 PFU IHD-J, and were monitored for weight (B) and survival (C). All of the animals progressively lost weight to 20% at day 4 p.c., with no relevant differences among the groups of vaccinated mice (B). From day 5 p.c., all of the animals of Group 4 (G4) started regaining weight and survived. 50% of the mice of Group 5 (G5) recovered and were protected after day 7 p.c. All of the animals of the other groups died between days 7 and 8 p.c. (C).

$5–30 \times 10^5$ PFU IHD-J died at day 6 post-challenge (p.c.), whereas all of the mice infected with 1×10^5 PFU IHD-J remained alive and developed strong immunity and protection against re-infection with high viral doses. When 2×10^5 PFU IHD-J were used, 50% of the mice survived (i.e., the LD_{50}, Fig. 6A) and were protected from high viral doses up to 5×10^6. The dose of 1×10^6 PFU IHD-J (5-fold the LD_{50}) was then chosen for the challenge. In the experimental challenge, soon after the first day p.c., all of the mice progressively lost weight, by 20% at day 4 p.c., with no relevant differences between the groups of vaccinated mice (Fig. 6B). At day 5 p.c., all of the mice of Group 4 started regaining weight and survived, whereas only 50% of the mice of Group 5 recovered after day 7 and survived (Fig. 6B and C). All of the mice of Groups 1, 2, 3, 6, 8 died between days 7 and 8 p.c.

4. Discussion

The development of safer vaccines against OPXV infection of humans is still an emerging concern that might arise either through accidental exposure to CPXV, MPXV or other OPXV (Reed et al., 2004), or following deliberate bioterrorist release of variola virus. The discontinuation of the smallpox vaccination campaign resulted in declined herd immunity, with increased MPXV zoonotic infections (Hutin et al., 2012), which has encouraged the development of safer smallpox vaccines (Artenstein, 2008; Poland, 2005; Wiser et al., 2007). Although with low frequency, serious side effects can result from live VV-based vaccines, especially in immunocompromised subjects and patients with skin diseases (Schulze et al., 2007). Highly attenuated MVA is thought to be safe (Kennedy and

Greenberg, 2009), although it requires 1000-fold the dose of the current live VV vaccine.

It is already well established that combined immunisation regimens where DNA priming is followed by protein or viral vaccine boosts elicit higher immune responses compared with the non-combined use of the immmunogens (Lu, 2009; Radaelli et al., 2003, 2007; Wang et al., 2008). In particular, heterologous DNA prime/protein boost strategies have been effective in eliciting improved quality of antigen-specific antibody responses (Vaine et al., 2010), and thus this approach might offer new possibilities to develop safe and improved vaccines.

In the present study, four new FP recombinants, FP_{L1R}, FP_{A27L}, FP_{A33R} and FP_{B5R}, were combined with DNA recombinants, all of them expressing the VV *L1R, A27L, A33R* and *B5R* genes, and were administered as novel homologous/heterologous prime/boost immunisation regimens. Our aim was to evaluate the humoral and cell-mediated responses as well as the protection in mice, through the comparison of different vaccination protocols. We have demonstrated that: (i) the specific antibody responses that were elicited by the different protocols do not correlate with protection; (ii) the presence of neutralising antibodies in the serum does not always allow mouse survival; (iii) the putative protective role of the cellular responses does not always occur, as shown by IFN-γ/IL4 cytokine determination as a surrogate for the Th1/Th2 responses.

Although still controversial, the critical role of the antigen-specific humoral response for vaccine-induced protection against OPXV has already been described (Edghill-Smith et al., 2005b; Panchanathan et al., 2006; Sarkar et al., 1975). In particular, while CD8+ T cells appear not to be necessary for protection from a lethal MPXV challenge (Edghill-Smith et al., 2005b), passive transfer of VV-specific sera was shown to confer protection both in mice and monkeys (Golden et al., 2011). Vaccination with live VV appears to be effective also in subjects with dysfunctions in their humoral response, although not in subjects with T-cell related immunodeficiencies (Golden and Hooper, 2013).

In the present study, although high variability was found among the animals which might weaken the results, the highest level of humoral response was obtained both after the second and third immunisation for the mice of Group 3, which received the 4FPmix repeatedly. However, none of these animals survived the challenge, although antibodies showed virus neutralising activity. Conversely, the mice of Group 4 showed highly significant humoral responses and were protected. In Group 5, only 50% of the mice survived, in spite of their VV-specific antibody response. The neutralisation of infectivity, which generally correlates with the level of antibodies against virus surface antigens, was significantly higher in the mice of Group 4 that were all protected, but remained low in the mice of Group 5. Neutralisation was thus performed separately on the sera from each mice of Group 5 to determine whether the neutralising activity correlated with the protection. Surprisingly, the significantly higher level of neutralising antibodies of the protected mice 4 and 7 was not confirmed by mouse 5, which was also protected, but which showed lower neutralising antibody levels than the unprotected mice (mice 1, 2, 8). This suggests that protection cannot be ascribed only to the level of neutralising antibodies.

Although the protective role of CTL after OPXV vaccination remains to be defined (Buchman et al., 2010), immunogens that target T-cell epitopes also appear to be effective (Goulding et al., 2013; Hirao et al., 2011; Moise et al., 2011; Snyder et al., 2004). As specific cytokine release is one of the main characteristics of the Th1/Th2 responses, both IFNγ and IL4 production were assessed. IL4 production varied only slightly among the groups, whereas the number of IFNγ-producing cells was higher in the mice of Group 4, which resulted in a significantly higher IFNγ/IL4 ratio in protected mice. When testing the effector function of specific CD8+ T-cells, similar higher cytotoxic activity was found in the spleen of the mice

of Groups 4 and 5. This suggests a pivotal role of IFNγ-producing T cells and cytotoxic cells in the mice of Group 4, which is also in agreement with other recent studies that have underlined the need for T-cell responses for survival after VV challenge.

Although the weight decrease soon after the challenge was similar in all of the immunised groups, it is surprising that this loss was consistently almost 5% higher in the mice of Group 4, which then progressively regained weight at day 5 p.c. and were protected. As three out of six mice of Group 5 also regained weight at day 7 p.c. and were protected, these results suggest a transient negative effect on animals vaccinated with MVA, and confirms that MVA can provide 100% protection only after repeated administrations (Handley et al., 2009; Kennedy and Greenberg, 2009; Kenner et al., 2006). Although the use of a different vector might avoid the neutralisation elicited by MVA priming, the boosting with FP-based recombinants did not elicit protection in all of the animals of Group 5.

In spite of the evidence from other studies, DNA recombinants alone did not provide any protection. This might be ascribed to the absence of repeated DNA immunisation or to a different way of DNA administration. It is well known that a more effective immune response can be obtained by heterologous prime/boost, although the route and dose of vaccine administration might also be determinants (Bansal et al., 2008). This is further supported by a recent study in which an optimal dose of the VV Tiantan attenuated strain was used, with higher levels of long-lasting neutralising antibody responses induced in protected mice through the intranasal/oral than the i.m. and s.c. routes (Lu et al., 2011). If FP evasion mechanisms can be hypothesised that can inactivate cytokines, these mechanisms were not active in previous immunisation experiments in which other FP recombinants were used, and it prompts us to improve immunogenicity by different routes of immunisation.

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