

# Enhancing poxvirus vectors vaccine immunogenicity

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**A**ttenuated recombinant poxvirus vectors expressing heterologous antigens from pathogens are currently at various stages in clinical trials with the aim to establish their efficacy. This is because these vectors have shown excellent safety profiles, significant immunogenicity against foreign expressed antigens and are able to induce protective immune responses. In view of the limited efficacy triggered by some poxvirus strains used in clinical trials (i.e., ALVAC in the RV144 phase III clinical trial for HIV), and of the restrictive replication capacity of the highly attenuated vectors like MVA and NYVAC, there is a consensus that further improvements of these vectors should be pursued. In this review we considered several strategies that are currently being implemented, as well as new approaches, to improve the immunogenicity of the poxvirus vectors. This includes heterologous prime/boost protocols, use of co-stimulatory molecules, deletion of viral immunomodulatory genes still present in the poxvirus genome, enhancing virus promoter strength, enhancing vector replication capacity, optimizing expression of foreign heterologous sequences, and the combined use of adjuvants. An optimized poxvirus vector triggering long-lasting immunity with a high protective efficacy against a selective disease should be sought.

**Keywords:** poxvirus, immunogenicity, vaccines, recombinants, vector improvements

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## Introduction

Vaccination is considered to be the most effective and less expensive strategy for the control and eradication of human diseases. While many infectious

diseases have been controlled through vaccination, however, there are others for which vaccines are not yet available. In fact, infectious diseases are responsible for more than 15 million deaths a year, and affect the health and life expectancy worldwide.<sup>1</sup> Furthermore, on a yearly basis about 2 million people died from human immunodeficiency virus (HIV), 1.5 million from tuberculosis, 700 000 from malaria, 12 million are infected with leishmania species, and 170 million are infected with hepatitis C (HCV), to cite a few examples. Moreover, cancer with an estimated 13.1 million deaths in 2030, as well as diseases of the elderly, like Alzheimer and Parkinson, for which effective treatments are not available, are being approached by vaccination. The difficulties encountered in vaccine development are largely due to a lack of understanding of the pathology of the microorganism, the virus-host interaction, the immunogen design, the immunization protocols, and the definition of immune correlates of protection. With the new technological advances derived from system biology and immunological approaches, now the way we look at vaccination has changed, and the finding of vaccines that could control the impact of these infectious diseases is one of the main priorities in the research field. However, the complexity of the pathogens, the co-infections, and the emergence of new drug resistant microbes highlight the need of new antimicrobial agents based on innovative therapeutic strategies, new vaccines, and other preventive approaches.

Poxviruses belong to the Poxviridae family and to the Chordopoxvirinae subfamily, and contain a double-stranded DNA genome. One of the main

characteristics of these viruses is that large foreign DNA sequences can be inserted in their genome, allowing their use as vectors for heterologous gene sequences which encode antigens derived from several pathogens (such as parasites, bacteria, and viruses), tumor cells, or other foreign sequences. Therefore they are ideal vectors to be used as recombinant poxvirus vaccines. Moreover, there are several advantages in the use of poxvirus vectors as vaccines: (1) packing flexibility of the genome, (2) lack of persistence or genomic integration in the host due to their cytoplasmic replication, (3) ability to induce both antibody and cytotoxic T cell responses against the heterologous antigens with long-lasting immunity, (4) thermostability of freeze-dried vaccine, low cost, ease of manufacture and administration, and (5) low prevalence of anti-vector immunity in the global population due to the interruption of smallpox vaccination in the 1970s following its eradication. Since in the early 1980s recombinant vaccinia virus (VACV) strains expressing foreign genes were constructed,<sup>2,3</sup> poxviruses have played a main role as candidate vector systems for vaccination against a broad spectrum of infectious diseases and several recombinant poxvirus vaccine candidates have been successfully assayed in preclinical and clinical trials using homologous or heterologous combinations in the prevention and treatment of different human and animal diseases.<sup>4-17</sup> These vectors provide unique forms of viral vaccines that combine the safety of a killed virus vaccine due to their impaired replication capacity with the immunogenicity of a live virus vaccine by expressing gene products within cells that are efficiently presented by both major histocompatibility complex (MHC) class I and class II pathways, leading to the activation of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells, to the induction of antigen-specific antibodies and to the activation of the innate immune responses.

Some attenuated poxvirus vectors, like ALVAC, modified vaccinia virus Ankara (MVA), NYVAC, and Fowlpox, are the most promising vectors and they have entered into a large number of clinical trials with some of them rapidly moving forward into more advanced prophylactic and therapeutic clinical trials as vaccines

against many diseases. However, despite the good safety and immunogenicity profiles exhibited by these attenuated poxvirus recombinant vectors, novel optimizing more efficient poxvirus vectors with an enhanced magnitude, breadth, polyfunctionality, and durability of the immune responses to exogenously expressed antigens are desirable, and further improvements of these vectors should be pursued. Preclinical trials in animal models (such as mice and non-human primates) are essential to evaluate the safety and immunogenicity of these novel optimizing poxvirus vectors, and the results obtained will give important information to choose the best-in-class vaccine candidates for evaluation in future human clinical trials. Moreover, the animal studies will provide relevant insights in the immune correlates of protection. Thus, an optimized poxvirus vector inducing long-lasting immunity with a high protective efficacy should be sought.

In this review we describe several approaches for vaccine improvements of poxvirus vectors, with particular emphasis in the 2 candidate vaccine vectors most extensively used, MVA and NYVAC. Clinical application of these vectors as vaccines are considered in other reviews.<sup>4,6-15</sup>

## Vaccine Improvements of Poxvirus Vectors

### Heterologous prime/boost protocols

One of the strategies experienced over the last decades to increase the immunogenicity of poxvirus vectors is to combine the vaccines in "prime-boost" vaccination regimens. We, in collaboration with other colleagues, pioneered a prime/boost protocol to enhance specific CD8<sup>+</sup> T cell immune responses with poxvirus vectors, revealing the order of heterologous vector delivery and immune requirements for protection after challenge in the murine malaria model.<sup>18-20</sup> These heterologous prime/boost protocols when combined with a poxvirus vector are now widely used in many preclinical and clinical trials.<sup>4,6-15</sup> Most of the clinical trials used a poxvirus vector in combination with other vectors and the findings thus far are consistent with: (1) the use of a poxvirus as a booster component to enhance the magnitude

of the antigen-specific T cell responses; (2) as a priming component in combination with a protein plus an adjuvant to enhance B cell responses and trigger effective antibody responses to the foreign antigen. As an example, the use of MVA recombinants as the boosting immunogen in combination with different vectors such as recombinant influenza virus,<sup>21</sup> adenoviruses,<sup>22-26</sup> DNA vectors<sup>27-29</sup>, or other poxvirus strains<sup>18,19,30</sup> has proven to be an excellent regimen to elicit antigen-specific immune responses and, more importantly, to trigger protection against different pathogens.

### Use of co-stimulatory molecules

The purpose of this strategy is to improve the immunogenicity and efficacy of poxvirus vectors by the simultaneous co-delivery within cells of cytokines/chemokines together with the expressed foreign antigen. This has been accomplished either through genomic integration of the gene encoding the immune-stimulating molecule under control of a virus promoter or by the simple exogenous inoculation of soluble molecules in an organism. Thus, numerous studies have shown that insertion into the poxvirus vector genome or exogenous inoculation of some co-stimulatory molecules, such as interleukin (IL)-12,<sup>31-37</sup> interferon (IFN)- $\gamma$ ,<sup>31,38</sup> IL-2,<sup>39-41</sup> IL-15,<sup>42-47</sup> OX40/OX40L,<sup>48-50</sup> a triad of B7-1, ICAM-1, LFA-3 molecules,<sup>49,51-57</sup> a triad of CD80, CD86, and CD83 molecules,<sup>58</sup> CD40L,<sup>59-62</sup>, or GM-CSF<sup>36,63-67</sup> significantly enhances the immunogenicity and efficacy of the poxvirus vector as vaccine against different diseases. Some of these molecules are now being tested with poxvirus vectors in clinical trials.<sup>10</sup>

### Deletion of viral immunomodulatory genes still present in the poxvirus genome

While studies on how the vector impacts on the cell have progressed significantly over the last few years and an important knowledge is rapidly emerging on the signaling pathways and on host immune responses to the vector, however, less is known on how the vector impacts tissues in an organism and the consequences for activation of host immune responses. This behavior will be conditioned by the influence of viral immunomodulatory genes expressed from the poxvirus genome.

Thus, a complementary approach that is currently assayed to enhance the immunogenicity of the poxvirus vectors is the deletion of viral immunomodulatory genes, that are still present in the poxvirus vector genome, and whose gene products may be predicted to interfere with the optimal induction of cellular and humoral responses triggered by the host.<sup>8,68-70</sup> This strategy has been extensively developed during the last years in MVA and NYVAC recombinants used as vaccine candidates against HIV/AIDS, where different vector-based HIV vaccines with deletions in single and multiple immunomodulatory VACV genes which antagonize host specific immune responses have been generated. The overall results obtained in preclinical studies in mice<sup>71-79</sup> and non-human primates<sup>80,81</sup> showed a significant immunological benefit with an enhancement in the immunogenicity against HIV antigens compared with their parental poxvirus-based HIV vaccines. For example, a single deletion in the MVA vector of VACV genes encoding inhibitors of type I IFN signaling pathway (*C6L*),<sup>76</sup> apoptosis (*FIL*),<sup>79</sup> IL-18 binding protein (*C12L*)<sup>72</sup> or an inhibitor of IRF-3 (*N2L*),<sup>74</sup> enhanced the overall immune responses to HIV-1 antigens, with an increase in the magnitude, polyfunctionality, and durability of HIV-1-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However, when additional deletions were introduced in the MVA vector, the antigen-specific immune responses triggered were variable, either enhanced or suppressed. On one hand, responses against HIV-1 antigens were further enhanced using MVA vectors with 2 VACV gene deletions, *A41L/B16R*<sup>75</sup> or *C6L/K7R*.<sup>73</sup> On the other hand, 4 deletions in MVA [IL-18 binding protein (*C12L*), Toll/IL-1 receptor homolog (*A46R*), CC-chemokine binding protein (*B7R*) and secreted IL-1 $\beta$  receptor (*B16R*)] enhanced by 6-fold the frequencies of HIV-1-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses, while an additional deletion of uracyl-DNA glycosylase (MVA101R) decreases the responses, as evaluated in macaques.<sup>80</sup> Interestingly, deletion of VACV genes in the vector backbone of an MVA vector expressing Env, Gag, Pol, and Nef from clade B (MVA-B) was able to induce a shift in the HIV-1 CD8<sup>+</sup> T cell immune responses

in immunized mice; with the parental MVA vector inducing mainly Env-specific CD8<sup>+</sup> T cell immune responses and the MVA-B deletion mutants inducing preferentially Gag-Pol-Nef-specific CD8<sup>+</sup> T cell immune responses, both in the adaptive and memory phases.<sup>73,75,76</sup> In addition, MVA-B deletion mutants induced an increase in the CD8<sup>+</sup> T effector memory cells,<sup>73-76</sup> a parameter which have been described to correlate with HIV-1 viral control in early and chronic infection in humans,<sup>82-84</sup> and on the early control of highly pathogenic SIV in non-human primates.<sup>85,86</sup> Furthermore, a novel MVA vector lacking the viral genes *C6L*, *K7R*, and *A46R* and expressing the chikungunya structural antigens has been shown to induce in mice potent B and T cell immune responses with full protection against challenge after a single virus vector dose.<sup>87</sup> Moreover, NYVAC vectors with a single deletion of a VACV gene encoding a TLR inhibitor (*A46R*),<sup>78</sup> and with a single or double deletions of VACV genes encoding type I and II IFN-binding proteins (*B8R*, *B19R*)<sup>77</sup> respectively, were also able to enhance the immune responses to HIV-1 antigens in mice.

From all these observations, it is clear the contribution of poxviral genes as antagonists of the immune system and that their removal results in an enhancement of the immune responses to specific foreign antigens delivered from the vector, particularly CD4<sup>+</sup> and CD8<sup>+</sup> T cells, with the latter being the dominant. Moreover, these immunological studies pointed out to the further improvement of the poxvirus vector as a vaccine candidate. Evaluating the role played by all of the poxvirus immunomodulatory genes, with the aim to develop optimal vectors for vaccine considerations, should be undertaken. Therefore, novel optimized poxvirus vectors with deletions in VACV genes that enhanced antigen-specific immune responses are potential vaccine candidates. Future preclinical trials in non-human primates and clinical trials in humans with these novel poxvirus deletion mutants are ongoing and necessary to test whether these recombinants promote a higher antigen-specific immune response that could correlate with protection, than parental vectors.

## Enhancing virus promoter strength

The purpose of this strategy is to optimize the virus promoter strength to improve the levels of the foreign antigen expressed by the recombinant poxvirus vector, and to express the heterologous antigen at very early times postinfection, because it has been shown that the levels of recombinant antigen expressed from poxvirus vectors correlates with the magnitude of the immune responses in mice,<sup>88</sup> and timing of expression influence the type (CD4<sup>+</sup> or CD8<sup>+</sup>), quantity and quality of the immune response.<sup>89-92</sup> Thus, the timing of expression of heterologous antigens in the VACV system affects the capacity to induce antigen-specific T cell immune responses<sup>89</sup> since the efficiency with which an antigen is processed and presented on the surface of infected cells influences its recognition.<sup>93</sup> Moreover, the transgene expression levels are also dependent on the route of immunization.<sup>94</sup> Considering that immunodominance is defined as the phenomenon whereby only a small fraction of all of the possible epitopes from a particular pathogen elicits an immune response in an infected individual,<sup>95</sup> it is possible to modulate such immunodominance hierarchy changing the timing and the quantity of intracellular antigen production.<sup>96</sup> In fact, it has been described that in VACV 90% of the most recognized antigens by CD8<sup>+</sup> T cells were ranked in the top of 50% in terms of mRNA expression<sup>97</sup> and there is a correlation between viral gene expression and immunodominance hierarchy after a second immunization due to a mechanism of cross-competition between T cells specific for early and late viral epitopes.<sup>98</sup> Recently, after a deep sequence analysis of VACV transcriptome, 2 groups have defined 2 categories of early genes based on their temporal expression.<sup>99,100</sup>

Poxvirus vectors promoters with both early and/or late activity are used to direct the expression of foreign antigens to ensure that adequate expression levels are present at the appropriate time for induction of strong immune responses. The VACV TK locus has been traditionally used for insertion of heterologous sequences,<sup>101</sup> although it has been reported the use of endogenous MVA promoters *in situ* (at their natural site in the MVA genome) by

the deletion of the downstream genes and the subsequent insertion of a transgene at the deletion site.<sup>102</sup> Promoter strength is variable and can be a constitutive early/late promoter, such as p7.5, mH5 and the short synthetic promoter PrS,<sup>102-105</sup> with exceptional strong intermediate/late promoters such as I1L.<sup>106</sup> The work in the identification of viral promoters able to accelerate foreign gene expression with time of infection is expanding, and different viral promoters are being used to enhance expression of genes of interest.<sup>89,102,103,107-109</sup> For example, to enhance the promoter strength, some promoters such as PrS were designed in silico based on early and late consensus motifs observed in native poxvirus promoters.<sup>103</sup> In addition, the hybrid early-late synthetic promoter pHyb has been shown to drive the expression of antigens earlier during infection when compared with the PrS and p7.5k promoters and also to induce stronger CD8<sup>+</sup> T cell responses after repeated vaccinations.<sup>89</sup> Also, it has been reported that a novel naturally occurring tandem promoter in MVA termed MVA13.5L drives very early gene expression and potent immune responses.<sup>109</sup> Moreover, we have recently described a new synthetic Late-Early Optimized (LEO) VACV promoter that was designed after bioinformatic analysis, and in vitro significantly enhanced the levels of the foreign antigen green fluorescent protein (GFP) within the first hour after infection, which correlates with an enhancement in the GFP-specific CD8<sup>+</sup> T cell immune response detected *in vivo* (mice) and compared with the commonly used synthetic early/late promoter, demonstrating its potential use in future vaccines.<sup>107</sup>

Thus, novel optimizing promoters represent excellent prototypes for future usage to potentiate the expression of antigens from different pathogens and to generate safe VACV recombinant based vaccines able to induce potent immune responses that prevent the development of the disease.

#### **Enhancing vector replication capacity**

This strategy involves the generation of new vectors with an attenuated phenotype and with replication competence in human cells in order to increase the

timing and level of expression of the heterologous antigen in the host. To date, some replication-competent recombinant VACV-based vaccines have been used for various infectious diseases, demonstrating that they are able to elicit potent humoral and cellular immune responses and to confer long-lasting protection while maintaining a safety profile.<sup>110-113</sup>

We have previously reported that insertion of the host range *C7L* gene into NYVAC recombinant vectors expressing HIV-1 antigens significantly improved the magnitude and quality of the HIV-specific immune responses in mice.<sup>112</sup> Moreover, restoration of replication competence in human cells of a NYVAC recombinant expressing HIV-1 antigens from clade C (NYVAC-C) by re-incorporation of the *K1L* and *C7L* host range VACV genes (NYVAC-C-KC) or combination of restoration of replication competence with removal of the immunomodulatory viral molecule B19 protein (NYVAC-C-KC-ΔB19R) increased the expression of the heterologous antigen in infected human cells. This in turns, elicits an enhanced cross-presentation to HIV-specific CD8<sup>+</sup> T cells and proliferation of HIV-specific memory CD8<sup>+</sup> T cells *in vitro*, and selectively activated IFN-induced genes and genes involved in antigen processing and presentation, as determined by microarray analysis of infected human dendritic cells (DCs), while maintained limited virus spread in tissues and an attenuated phenotype.<sup>114,115</sup> Other attenuated replication competent vectors are M65 and M101, derived from the Western Reserve (WR) strain after multiple passages in Friend erytroleukemia cells, that have been shown to contain mutations in multiple gene sequences but with the capacity to trigger immunogenicity and protection against leishmania parasitic infection.<sup>116</sup> Another replication-competent and attenuated smallpox-derived vaccines, such as the Japanese LC16m8 (derived from the Lister vaccinia strain),<sup>117-119</sup> and the Chinese Tian-Tan strains<sup>111,120,121</sup> are also being investigated as vaccines for other diseases. Moreover, myxomavirus have been used as a promising vaccine candidate against several types of cancer,

due to the oncolytic ability of this poxvirus to specifically infect various classes of human cancer cells.<sup>122</sup>

#### **Optimizing expression of foreign heterologous sequences**

In addition to the modifications of the vector backbone described above, another approach to improve the immunogenicity of a poxvirus vector is through optimization of the expressed antigen. This can be accomplished by codon optimization and protein design, although a rational design of the heterologous transgene inserted in the poxvirus vector has not been extensively reported. It has been shown that the insertion in the transgene of an N-terminal signal peptide enhanced antibody immunogenicity from recombinant VACV.<sup>123</sup> Furthermore, a recombinant VACV expressing mosaic immunogen sequences designed to represent all of the potential HIV-1-specific T lymphocyte epitopes, elicited CD8<sup>+</sup> T cell responses that confer enhanced immune coverage of diverse HIV-1 strains in monkeys,<sup>124</sup> showing that the mosaic immunogen induces greater cross-reactivity than the consensus immunogen. This increased breadth and depth of epitope recognition could contribute to protection against infection by genetically diverse viruses and, in some instances, may block the emergence of common variant viruses. Moreover, recently it has been described that poxvirus vector-based vaccines expressing HIV-1 mosaic Env, Gag, and Pol in combination with adenovirus vectors, afforded a significant reduction in the acquisition risk following SHIV-SF162P3 challenge, with protection against acquisition of infection correlating with vaccine-elicited binding, neutralizing, and functional non-neutralizing antibodies.<sup>125</sup> These data suggest that the coordinated activity of multiple antibody functions may contribute to protection against difficult-to-neutralize viruses, and demonstrate the protective efficacy of HIV-1 mosaic antigens.

Also, the use of conserved immunogens in prime-boost strategies has been established.<sup>126</sup> Additionally, in the case of MVA and NYVAC recombinants expressing HIV-1 antigens, we have developed different forms of Env and of Gag

antigens that have been engineered to produce cell-released products, as gp120, trimeric gp140, and of a polyprotein of Gag-Pol-Nef, either remaining intracellular or being released as Gag-derived VLPs.<sup>27,115,127</sup> These modifications have the added advantage of removing non-desirable sequences, to improve the stability of the recombinant vector and of the expressed antigen. Moreover, a cell-released product will be important for an optimal and higher induction of antibody responses. Mice immunized with these novel recombinants poxvirus vectors have shown enhanced HIV-1-specific immune responses,<sup>27,127</sup> reinforcing these new vectors as future promising components of an HIV/AIDS vaccine candidate. These MVA and NYVAC vectors have shown good immunological behavior in phase I clinical trials.<sup>128-132</sup>

#### Combined use of adjuvants

While poxvirus virions per se are potent immunogens, the vectors can themselves act as immunogens when combined with other antigens. In fact, the HIV RV144 phase III clinical trial<sup>133</sup> has shown that the combination of the poxvirus vector ALVAC expressing HIV-1 antigens and a purified HIV-1 gp120 is an effective approach to induce high affinity antibodies that correlate with protection.<sup>134-138</sup>

The experience accumulated thus far is that, some adjuvants when combined with different poxvirus vectors can enhance the antigen-specific immune responses. For example, the multimeric soluble CD40 ligand (sCD40L) efficiently enhanced HIV-specific cellular immune responses during DNA prime and boost with MVA and NYVAC expressing HIV-1 antigens,<sup>59</sup> and CD40L expressed from the canarypox vector, ALVAC, boost the immunogenicity of HIV-1 canarypox vaccine in mice and enhanced the in vitro expansion of viral specific CD8<sup>+</sup> T cell memory responses from HIV-1-infected and HIV-1-uninfected individuals.<sup>61</sup> Furthermore, genetic adjuvantation of recombinant MVA with CD40L potentiates CD8<sup>+</sup> T cell mediated immunity,<sup>60</sup> and oncolytic vaccinia virus coding for CD40L mediates multiple antitumor effects including oncolysis, apoptosis, and induction of Th1 type T-cell responses.<sup>62</sup> Recently, it has been described that Glucopyranosyl Lipid

A (GLA) adjuvant significantly enhances HIV-1-specific T and B cell responses elicited by a DNA-MVA-protein vaccine regimen.<sup>139</sup> Moreover, a MVA prime followed by either intranasal or systemic protein boosts together with the adjuvant MF59 can elicit strong humoral responses in breast milk and may be a useful strategy to interrupt postnatal HIV-1 transmission in non-human primates.<sup>140</sup> In addition, the use of the adjuvant IC31, a TLR9 agonist, in immunized mice greatly improves vaccination immunogenicity in terms of the development of Env-specific humoral and cellular responses, when combined with a NYVAC-CN54 expressing HIV envelope gp120.<sup>141</sup> Other adjuvants, like poly I:C are also being tested in preclinical trials in combination with poxvirus vectors.<sup>142</sup> In addition, targeting HIV-1 proteins to dendritic cells by fusion with specific cell receptors followed by a poxvirus boost is another encouraging strategy to enhance HIV-specific immune responses.<sup>142</sup>

Moreover, oligomeric fusion proteins act also as stronger immunogens than single components. In fact, chimeras between VACV proteins and HIV Env had shown enhanced B and T cell responses to HIV-1 antigens.<sup>143</sup> In this way, we have recently described a fusion protein based on the VACV A27 protein (14 kDa) fused together with the circumsporozoite protein of Plasmodium (CS-14K). This fusion protein forms stable oligomers, was produced in quantities as a soluble product in *E. coli*, have an adjuvant-like effect, and when administered to mice in the absence of adjuvant and in combination with MVA expressing the CS protein of *P. yoelii* (MVA-PyCS), induced sterile protection after challenge with sporozoites of *P. yoelii*.<sup>144</sup>

#### Concluding Remarks

Since the demonstration by Jenner in 1796 that effective vaccination against smallpox was possible with a poxvirus vector, the declaration of eradication of this deadly disease by WHO in 1980 and the discovery of insertion of foreign genes encoding antigens in the genome of the poxvirus vector in 1982, with wide consequences on its use as vaccines against

multiple pathogens, a burst of scientific publications have been produced since them highlighting the potential clinical benefits of the recombinant poxvirus vectors as vaccines. While different poxvirus vectors have been or are currently being tested as human vaccines, there is as yet no recombinant poxvirus vector approved for use as human vaccine, with the exception in the veterinary field where several poxvirus vector vaccines are commercially available in the market. However, in light of the approval in Europe and Canada of the non-recombinant poxvirus vector MVA as a preventive vaccine for smallpox, and of the numerous clinical trials with attenuated poxvirus vectors for viral, parasitic, and tumor diseases, a major emphasis in the vaccine field is to optimally apply the newly generated vectors as immunogens. Different strategies have been followed, mainly, the heterologous prime/boost combination between vectors. From the lessons learned in the HIV RV144 phase III clinical trial, and on the use of highly attenuated replication restricted vectors like MVA and NYVAC, it is clear that the vectors currently being tested are not optimal. This is because the poxvirus genome contains multiple genes that antagonize the immune responses.

With the aim to optimally design a pox vector with enhanced ability to trigger B and T cell immune responses to foreign antigens superior to the current vectors, in this review we proposed several strategies in the rational design of poxvirus vectors that include, prime/boost protocols, use of co-stimulatory molecules, deletion of viral immunomodulatory genes still present in the poxvirus genome, enhancing virus promoter strength, enhancing vector replication capacity, optimizing expression of foreign heterologous sequences, and the combined use of adjuvants. These strategies can be applied to any particular poxvirus strain, whether is replication restricted or replication competent. The implementation of any of these strategies will be dictated by the final aim to be pursued, that is, an optimized poxvirus vector triggering long-lasting immunity with a high protective efficacy against a specific disease.

Undoubtedly, for the better design of vaccines based on poxvirus vectors, we still

need to know many fundamental aspects of the biology of the vectors and their interaction with the host immune system. The implementation of system biology, proteomics, bioinformatics, and imaging will help to unravel immune mechanisms related to protection. An understanding of the role played by the different viral genes with immunosuppressive properties will be critical in the better design of poxvirus vector vaccines with a major impact on the immune system. Scientific discoveries from studies of the immune biology of this class of DNA vectors and correlates of protection, will gradually be made in the years ahead.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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