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Use of functional genomics to understand replication deficient poxvirus-host interactions.

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Abstract

High-throughput genomics technologies are currently being used to study a wide variety of viral infections, providing insight into which cellular genes and pathways are regulated after infection, and how these changes are related, or not, to efficient elimination of the pathogen. This article will focus on how gene expression studies of infections with non-replicative poxviruses currently used as vaccine vectors provide a global perspective of the molecular events associated with the viral infection in human cells. These high-throughput genomics approaches have the potential to lead to the identification of specific new properties of the viral vector or novel cellular targets that may aid in the development of more effective pox-derived vaccines and antivirals.

1. Introduction

The eradication of Smallpox, due to the elimination from nature of the causal agent of small-pox disease, Variola virus, was an important milestone in the history of medicine (Fenner, 1977; Fenner, 1980; Fenner, 1982). This goal was achieved after a global vaccination campaign using Vaccinia virus (VACV), a virus that is antigenically similar to Variola virus (Fenner, 1982; Smith, 2013). The efficacy of poxvirus vaccination was due to their immunogenic properties, including the ability to induce long-term humoral and cell-mediated immunity (Crotty et al., 2003). However, the vaccination campaign for the elimination of smallpox also revealed a significant incidence of complications, after immunisation with wild-type VACV, that ranged in severity from benign to lethal, especially in those individuals with reduced immune function (Bray, 2003). Thus, much subsequent research on VACV has focused on producing modified vaccines with improved safety profiles (Moss, 2013). Two main types of approaches have been taken to enhance the safety of VACV (Esteban, 2009; Garcia-Arriaza and Esteban, 2014; Sanchez-Sampedro et al., 2015). A first, more empirical strategy, consists of successive passage of the virus in an unnatural host or in tissue culture, and the isolation of virus variants (Gomez et al., 2009). The alternative approach, based on our increasing knowledge of the biology of the virus, includes the deletion of specific viral genes involved in important host interactions functions (Gomez et al., 2012b) (Paoletti et al., 1995). Both approaches have led to safe VACV strains.

Modified Vaccinia virus Ankara (MVA), derived from the parental Chorioallantois Vaccinia virus Ankara (CVA) by more than 570 passages in chicken embryo fibroblast (CEFs) cells, is now considered an attractive and promising candidate viral vector for the expression of foreign genes of interest because its unique properties (Antoine et al., 1998; Gomez et al., 2013; Meyer et al.,

1991; Sutter and Staib, 2003). In particular, MVA, due to its avirulence and inability to replicate productively after *in vivo* inoculation, has a better safety profile than replication competent VACV, with similar levels of gene expression and better immunostimulatory properties (Antoine et al., 1998; Gomez et al., 2013; Meyer et al., 1991; Verheust et al., 2012). These characteristics have stimulated the development of MVA-based vaccines for a wide range of pathogens including malaria, leishmania (Moss, 1996; Schneider et al., 1998) and HIV, as well as for the treatment of cancer acting as a vector for delivery of effector molecules against tumors (Cebere et al., 2006; Corona Gutierrez et al., 2004; Gilbert et al., 2006; Harrop et al., 2006; Smith, 2013). MVA is also a potentially safe vaccine candidate for smallpox, in the hypothetical case of a recurrence of the virus as a bioterrorist weapon (Belyakov et al., 2003; Drexler et al., 2003; Earl et al., 2004; Wyatt et al., 2004).

Another virus generated by serial passages in CEFs is ALVAC, a plaque-purified clone derived from an attenuated canarypox virus (Plotkin et al., 1995). ALVAC differs significantly from MVA in terms of genome size (approximately 365 kbp versus approximately 178 kbp, respectively) and in the number of open reading frames (ORFs) (McFadden, 2005). Many of ALVAC's ORFs may not be functional in mammalian cells, as evidenced by the inability of avipoxviruses to replicate in mammalian cells, and this characteristic may underlie its improved safety profile as a vaccine vector (Meyer et al., 1991; Taylor et al., 1988). ALVAC has been extensively evaluated in preclinical studies with both humans and non-human primates and widely used in human in a phase III clinical trial as an HIV/AIDS vaccine candidate in Thailand (Rerks-Ngarm et al., 2009).

The generation of attenuated viruses by the deletion of specific viral genes is based on the observation that many poxvirus genes are dispensable for growth *in vitro*. For VACV this approach has involved the deletion of viral genes that modulate the immune response, host-range and metabolism and has been extended to include the deletion of genes essential for viral replication by the use of complementing cell lines expressing the targeted VACV gene. The deletion of single immunomodulatory VACV genes from different strains has frequently led to attenuation of the virus as demonstrated in mice, sometimes accompanied by an increase in the immunogenicity of VACV antigens, as has been described for the VACV genes *E3L* (Beattie et al., 1996) (Jentarra et al., 2008)), *B15R/B16R* (Staib et al., 2005), *A41L* (Clark et al., 2006), *B13R* and *A35R* (Rehm and Roper, 2011) or *C6L* (Sumner et al., 2013).

79 However the deletion of multiple viral genes can further enhance the attenuation and improve the
80 safety of VACV as has been demonstrated by the engineering of the NYVAC strain of VACV
81 (Brockmeier et al., 1993; Konishi et al., 1992). NYVAC is an attenuated derivative of the VACV
82 strain Copenhagen (CopV), produced by the specific deletion of 18 ORFs, involved in host range,
83 virulence and pathogenesis, from the genome of the parental virus (Tartaglia et al., 1992). Despite
84 its limited ability to replicate productively in human and most mammalian cells, NYVAC provides
85 a high level of foreign gene expression and efficiently triggers specific immune responses to these
86 antigens in both experimental animals and humans. NYVAC-derived vectors are able to express
87 antigens from a broad range of species (Tartaglia et al., 1992) and have been used as recombinant
88 vaccines against numerous pathogens and tumors (Franchini et al., 2004; Kanesa-athan et al.,
89 2000; Myagkikh et al., 1996; Sivanandham et al., 1998) and it has shown strong and specific
90 immunogenicity and a good safety profile in Phase I/II clinical trials against HIV-1 (Ockenhouse
91 et al., 1998).

92 The attenuated MVA, NYVAC and ALVAC strains of poxvirus have become attractive vaccine
93 vectors against HIV/AIDS. The arguments in favour of the use of these viruses as vaccine vectors
94 include excellent immunogenicity and safety profiles and limited pre-existing immunity to
95 poxvirus in the population at risk of HIV infection due to the abandonment of vaccine campaigns
96 after the eradication of smallpox in the 1970s. The global HIV pandemic is not yet under control
97 despite the reported recent decline in incidence (UNAIDS, 2013). According to the UNAIDS
98 report for the year 2014, more than 35 million people live with HIV in world, with 2.1 million new
99 infections each year, so that 69% of all people from sub-Saharan Africa live with HIV leading to
100 1.5 million deaths that can be attributed to HIV annually (UNAIDS, 2014). The effectiveness of
101 the currently available HIV preventive and control interventions depends on strict adherence to a
102 complex protocol (Abdool Karim et al., 2010; Cicconi et al., 2013; Vermund et al., 2013) with a
103 threat of recidivism (Kreek, 1974). The search for an HIV vaccine during the past 25 years has
104 been a challenge due to viral diversity and the ability of cells persistently infected to evade the
105 immune system (Saunders et al., 2012). However, pre-clinical studies have identified immune and
106 genetic biomarkers associated with protection against challenge that provide further insights for
107 an HIV preventive vaccine for humans (Barouch et al., 2012). So far, there have been more than
108 180 clinical HIV-1 vaccine trials conducted in humans ranging from phase I to phase III (Garcia-
109 Arriaza and Esteban, 2014; O'Connell et al., 2012; Vermund et al., 2013), including the recently

concluded RV 144 phase III trial in Thailand using canarypox that showed a modest efficacy of 31% (Rerks-Ngarm et al., 2009). Given the biomedical importance of achieving a vaccine against HIV, a section of this review will focus on studies using genomic poxvirus vectors as vaccine against HIV.

The increasing use of high-throughput technologies is leading to an ever-more detailed view of virus-host interactions (Law et al., 2013; Peng et al., 2009; Tan et al., 2007; Tree et al., 2014). However, as the almost overwhelming amount of information from these “omics” experiments present in the databases grows, the choice of which elements of these vast amounts of data to investigate further becomes more difficult, but more important. In this regard, several examples of detailed studies of the biological relevance of a selected gene regulation after infection performed as follow-ups to microarray experiments have been carried out (Guerra et al., 2005; Guerra et al., 2008) (Caceres et al., 2013). The aim of this article is to illustrate how data obtained from global gene expression experiments can be used to gain new insights into virus-host interactions, with particular emphasis on the immune response and its modulation by non-replicative poxvirus infection. The successful integration of this kind of information into attempts to understand biological processes at a systems level will be critical to the development of new vaccine vectors.

2. Overview of comparative genomic experiments performed with attenuated non-replicating poxvirus

2.1. Host gene regulation after MVA infection analysed by comparative genomic analysis.

Many aspects of the biology of MVA remain only poorly understood, and a deeper understanding of the genetic factors that influence poxviral replication and viral gene function is needed to permit further optimization of the safety and immunogenicity of MVA derived vectors. It seems reasonable to suggest that comparative genomic experiments will be an important source of increased knowledge of the impact of MVA infection on human cells (Guerra et al., 2004; Guerra et al., 2007; Royo et al., 2014).

The first experiments of comparative genomics using MVA virus were performed in HeLa cells, a cell line used in many key poxvirus biology studies because its high susceptibility to infection (Guerra et al., 2004). Cells were infected at 5 plaque-forming units (PFU) per cell to guarantee synchronous infection of all cells (Guerra et al., 2004), and the relative abundance of specific mRNAs were compared in MVA-infected cells and mock-infected cells at 2, 6 and 16 hours

postinfection (hpi). Compared to previous experiments using the same approach, but with the WR strain of vaccinia (Guerra et al., 2003), MVA infection was found to modulate a large number of immunomodulatory genes. Specifically, the transcription of several genes involved in the immune response (around 10 transcripts) was activated by MVA infection; six cytokines, *interleukin (IL) 1A (IL-1A)*, *IL-6*, *IL-7*, *IL-8*, and *IL-15* and five members of the *tumor necrosis factor receptor superfamily* *TNFRS*, *TNFRSF7*, *TNFAIP3*, *TNFRSF14*, *TNFRSF17*, and the *TNF receptor associated factor* (TRAF) 3 (*TRAF3*) (Guerra et al., 2004). These data indicated that, in contrast to WR infection, MVA infection was associated with a more pronounced antiviral immune response of the host cell rather than a process beneficial for viral replication.

A clear upregulation of *nuclear factor- κ B (NF- κ B)* gene expression (mRNA and protein) was also a feature of MVA infection in HeLa cells (Guerra et al., 2004). NF- κ B regulates more than 800 well-known cellular genes (Perkins, 2007) and is known to be important in inflammatory and immune responses, in the processes of proliferation and cell death in viral replication, and in the production of nitric oxide. Increased levels of NF- κ B expression combined with increased transcription factor activity may be the cause of the specific increase in expression of cellular genes regulated by NF- κ B after MVA infection (Guerra et al., 2004). These findings are consistent with previous data indicating an increase in NF- κ B activity in extracts from MVA-infected 293 cells due to the absence of *K1L* gene in the MVA genome (Oie and Pickup, 2001). The group of Oie and Pickup also identified that NF- κ B activation after MVA infection depends on a double-stranded RNA-activated protein kinase (PKR)-dependent pathway triggered during the early phase of virus replication (Lynch et al., 2009). It has also been shown that early MVA gene expression rapidly induces phosphorylation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 2 (ERK2), an event that precedes NF- κ B activation (Martin and Shisler, 2009).

A key requisite for the development of therapeutic applications based on VACV is detailed basic knowledge of the intracellular mechanisms which allow activation of the immune response. Since dendritic cells (DCs) and macrophages play critical roles in establishing immunity, it is essential to know the molecular events that occur during the infection of these cells with the different strains of VACV employed as vaccines. To study these aspects of MVA as a vaccine vector, comparative genomic experiments were done using human DCs (Guerra et al., 2007) and macrophages (Royo et al., 2014). In both cases, exposure to MVA led to an abortive infection and considerable

morphological damage at late times of infection associated with inhibition of protein synthesis, the degradation of ribosomal RNA and cell death or apoptosis (Guerra et al., 2007) (Royo et al., 2014). Further, an inhibition of expression of specific late viral proteins was detected after the infection of DCs or macrophages by MVA. Thus, for the genomic studies cells were infected at 5 PFU and analysed at 6 hpi, sufficient time to allow the establishment of infection and progression to late viral gene expression but avoiding the risk of cell lysis and RNA degradation. Gene-profiling experiments showed a significant increase in the transcription of proinflammatory cytokines (*IL-6*, *CXCL2*, *TNF- α*), type I IFN (*IFN- α/β*) and IFN stimulated genes (*IFIT1*, *IFIT4*, *ISG15*, *ISG20*, *OASL*, among others) in DCs infected with MVA. Moreover, DC maturation markers were also activated, for example, the infected-DCs upregulated expression of *the chemokine (C-C motif) ligand 2 (CCL2)* gene that produces an induction in the chemotaxis of monocytes and promotes migration of leukocytes to the lung (Lehmann et al., 2009). Thus increased DC maturation and migration to the lymph node of may well underlie the high immunogenicity of antigens expressed by MVA. Similarly, it has recently been shown that MVA-infected DCs efficiently present MHC class II-binding peptides, including epitopes from endogenous viral proteins, to efficiently activate CD4⁺ T-cells (Thiele et al., 2015), which will also be advantageous for the use of MVA as a vaccine vector.

In these studies, the differences between host gene expression levels in DCs and HeLa cells infected with MVA were also compared (Guerra et al., 2007). Genes analysed in 6-hpi MVA-infected HeLa cells were selected based on the genes regulated by MVA in infected DCs and only those genes showing at least two fold-higher expression levels in one cell type than the other were considered. 34.5% of the genes selected were differentially expressed after MVA infection of DCs compared to HeLa cells indicating that MVA affects host gene expression differently depending on the cellular type. On the other hand, several genes upregulated in MVA-infected DCs also showed higher expression levels in MVA-infected HeLa cells. These include *TNF α* , *IFN- β* , *IFN-induced protein with tetratricopeptide repeats 4 (IFIT4)* and *1 (IFIT1)* genes and chemokines such as *CXCL2*. A upregulation of two members of the cytosolic pattern recognition RLR receptors, *retinoic acid-inducible gene 1 (RIG-I)*, (also known as *DDX58*) and *melanoma differentiation-associated gene 5 protein (MDA5)* (also known as *helicard*) was also observed. The protein products of both these genes function as sensors of RNA viruses (Hornung et al., 2006; Kang et al., 2002; Kato et al., 2006; Pichlmair et al., 2006; Yoneyama et al., 2004); RIG-I detects the 5'-

triphosphate modification of ssRNAs and short dsRNAs, while MDA5 preferentially recognizes long dsRNAs (Hornung et al., 2006; Kang et al., 2002; Kato et al., 2006; Pichlmair et al., 2006; Yoneyama et al., 2004). Several studies have been carried out to investigate which VACV viral gene(s) influence cytosolic pattern recognition RLR receptors and which cellular pathways are involved (Dai et al., 2014). Specifically, using murine primary keratinocytes it has been shown that E3 could modulate immunity triggered by MAVS-dependent pathways (Deng et al., 2008), while the viral gene C16 controls the DNA-PK DNA sensor (Peters et al., 2013).

In other studies, MVA infection of monocytes, DCs and B-cells showed that monocytes were activated strongly after the infection, with upregulation of co-stimulatory molecules, *MHC molecules* and the cytokine *chemokine (C-C motif) receptor 7 (CCR7)* (Flechsigs et al., 2011); in contrast, infected immature DCs (iDCs) showed only partial activation; and infected B-cells were inhibited. Moreover, while expression of cytokines including *CXCL10*, *TNF- α* , *IL-6* and *IL12-p70* was enhanced, *IL-1 β* and *IL-10* levels were unaltered or even downregulated. MVA infection was associated with a high rate of apoptosis of antigen-presenting cells (APC), however, incubation of MVA-infected leukocytes with uninfected iDCs leads to complete maturation of the DCs and may be the basis for cross-presentation of MVA-encoded antigens. This approach seems to be an ideal model for further studies with MVA-encoded viral antigens regarding immunotherapy and vaccination strategies.

Another feature common to HeLa cells infected with MVA (Guerra et al., 2004), NYVAC (Guerra et al., 2006b), VV Δ E3L (Langland et al., 2006) and MVA-infected DCs was a clear upregulation of *IFN stimulated gene 15 (ISG15)*, an antiviral gene that controls macrophage phagocytosis after IFN production (Yanguéz et al., 2013). Similarly, the enhanced IFN signaling produced after MVA infection also stimulated the phagocytic potential of naïve macrophages (Royo et al., 2014). Since phagocytosis is important for both innate and adaptive immunity, its upregulation after MVA infection could influence the extent and quality of the protective host response after vaccination. IFN induction in MVA-infected macrophages is higher than that observed following infection of dendritic cells and over 400 times higher than seen in uninfected cells (Royo et al., 2014). This strong IFN induction produced by MVA infection of macrophages is probably one of the crucial factors that make MVA an excellent vaccine candidate. The elevated production of IFN after MVA infection could have other beneficial effects (in addition to increased phagocytosis) and may underlie the enhanced immunogenicity of MVA compared to other vectors. In addition, since the

use of MVA is completely safe in human inoculation (Kibler et al., 2011), it may even be feasible to use exposure to MVA as a substitute therapy for exogenous IFN in patient groups such as HCV-infected individuals (Fattovich et al., 1996), certain forms of cancer (Tomasello et al., 2014) and patients with multiple sclerosis and other diseases (Reder and Feng, 2014).

It should be pointed out that other authors have described that infection of human whole blood with MVA induced a clear upregulation of *IL-8*, *CCL3* and *CXCL10* and less abundant production of pro-inflammatory cytokines (*TNF- α* , *IL-1 β* , *IL-6*) (Delaloye et al., 2009). These data are in conflict with the previously described downregulation of *IL-8* and *IL-1 β* mRNA expression in MVA-infected DCs and macrophages (Guerra et al., 2007) (Royo et al., 2014), and may reflect that patterns of cytokine production after MVA infection may vary with the cell-type studied.

2.2. Differential gene regulation in macrophages infected with MVA versus the parental CVA

The genomes of poxviruses encode a large number of genes that actively interfere with the host response to infection, and particularly responses of the innate immune system including the production of IFN, activation of inflammasome, Toll-like receptor signalling pathways and the activation of complement (McFadden, 2005). The identities of some, but by no means all, of the viral genes that modulate these host responses are known and study of their mechanisms of action has provided many useful insights into the correct functioning of pathogen sensing in innate immunity. However, it is also clear that much remains to be learnt about how poxviruses modulate cellular and immune responses to infection and in this context comparative studies of infections with parental CVA and MVA seem likely to yield much useful information. Analyses of the genome sequences of MVA and CVA have revealed that the attenuated virus has suffered six large genomic deletions leading to the loss or truncation of 31 ORFs, and deletions, insertions and non-synonymous mutations are found in 122 of the 195 ORFs remaining in MVA when compared with their Ankara counterparts (Antoine et al., 1998; Meisinger-Henschel et al., 2007; Staib et al., 2004). These observations suggest that many of the viral gene products that modulate the host response to infection will have been lost or modified in MVA and this may explain the enhanced, compared to wild-type vaccinia, expression of genes encoding immunomodulatory molecules that is observed during MVA infection (Guerra et al., 2004; Guerra et al., 2007), but the nature and identity of the viral genes whose expression controls these differences are essentially unknown. Moreover, it is clear that the genome of MVA still conserves the possibility to express some viral

genes that act to interfere with immune system recognition of this virus (Antoine et al., 1998). The functional significance of these observations is unclear, but it is obvious that an increased understanding of this system may well allow manipulation of the viral genome to influence the nature and quality of the host response to infection. Comparison of the impact that infection with either MVA or the parental virus CVA has on human macrophages may help to optimize the generation of new candidate vaccine vectors.

In this section some unpublished comparative genomic studies of the cellular genes regulated after MVA infection compared with parental CVA virus and how these changes may relate to the attenuation of MVA during its derivation from CVA will be discussed. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors following standard protocols (Royo et al., 2014). Adherent monocytes were cultured for 7 to 10 days, to allow their differentiation into macrophages, and then infected at 5 PFU with either MVA or CVA. cDNA prepared from 6 hpi MVA-infected or 6 hpi CVA-infected macrophages was hybridized to SurePrint G3 Human Gene Expression Microarrays (Agilent) and the different patterns of gene expression were analyzed as described (Smyth and Altman, 2013). Using this approach, a number of up- and down-regulated genes were identified in the infected macrophages (**Table 1 and Supplementary 1**). These data were extended to identify pathways differentially modulated by MVA in comparison to CVA using the Ingenuity Pathways Analysis software (Figure 1) (Ingenuity® Systems, www.ingenuity.com). The top canonical pathways regulated in MVA vs CVA depicted in **Figure #1** can be classified into: (i) role of cytokines in mediating communication between immune cells, (ii) role of pattern recognition receptors in recognition of bacteria and viruses, (iii) altered T-cell and B-cell signalling and (iv) communication between innate and adaptative immune cells. When the networks regulated in MVA were analyzed (Figure #2) the more regulated networks focussed on the regulation of IFN- γ (Figure #2A) and TNF- α (Figure #2B), both of are implicated in “antimicrobial response, inflammatory response and infectious diseases”. The increased activation of a larger number of genes involved in the immune response may underlie the increased immunogenicity of MVA.

MVA infection modulated a larger number of immune stimulatory genes compared to CVA (Table 1), for example, the levels of *INF- α* , *β* and *γ* were clearly more upregulated in MVA-infected macrophages than those infected with CVA. This activation of IFNs was accompanied by a clear decrease in the expression of the IFN α/β receptor (*IFNAR*). In addition, 5 IFN-induced genes such

as *IFT2*, *IFT3*, *ISG20*, *MDA5*, *IFN-induced transmembrane protein 1 (IFITM1)* were also activated after infection with MVA (Table 1).

The expression of several other interleukins was also modulated after MVA infection compared to CVA: *IL-6*, *IL6-ST* and *IL-29* were induced, whereas *IL-10*, *IL-1A*, *IL-19*, *IL-23A*, *IL-1B* and *IL-12B* were downregulated as were the receptors for *IL-1* and *IL-7*.

The loss of different viral immunomodulators during the attenuation of the vaccine strain MVA probably underlies these alterations after infection with MVA compared to CVA (Price et al., 2013; Waibler et al., 2009). MVA has lost homologues of multiple genes encoding recognized poxvirus immune-evasion molecules, including the viral IFN type I and type II receptors (Meisinger-Henschel et al., 2007; Price et al., 2013; Waibler et al., 2009) and the viral serpin B13R (SPI-2) that is involved in the inhibition of the interleukin-1beta-converting enzyme that protects virus-infected cells from TNF- and Fas-mediated apoptosis (Kettle et al., 1997). The viral genes coding for the complement-binding protein secreted from infected cells to control complement activation (Uvarova and Shchelkunov, 2001), the viral semaphorin, the 35kDa chemokine-binding protein and the TNF α receptor are also deleted or fragmented (Alcami, 2003; Moss and Shisler, 2001). However, interestingly, some viral genes with immunomodulatory function have been maintained in the MVA genome and the possible effects of expression of these molecules on the efficacy of MVA-based vaccines remains to be determined. One of these conserved genes encodes the viral IL-1 β receptor (*IL1-Br/B16R*) (Antoine et al., 1998) whose expression in MVA-infected cells has been described ((Blanchard et al., 1998).

As mentioned above, a clear decrease in the expression of both IL-1 β and its cellular receptor is noted in macrophages infected with MVA compared to CVA, indicating that infection with MVA is associated with reduced levels of IL-1 β signalling. IL-1 β is a cytokine that plays an important role in the regulation of inflammatory processes and the host innate immune response against infectious agents (Sims, 2002) (Klekotka et al., 2010). IL-1 β production after MVA infection of macrophages is mainly stimulated by the TLR2-TLR6-MyD88, MDA-5-IPS-1 and NACHT Domain-, Leucine-Rich Repeat-, and PYD-Containing Protein 3 (NALP3) inflammasome pathways. NALP3 is a member of the nucleotide-binding oligomerization domain (NLR) family of receptors (Delaloye et al., 2009), involved in the sensing of both DNA (*papillomavirus*) and RNA (*retrovirus*, *hepacivirus*, *orthomyxovirus*) viruses (Burdette et al., 2012; Muruve et al., 2008; Niebler et al., 2013; Pontillo et al., 2012). NALP3 forms a multimeric cytosolic molecular complex

known as the NALP3 inflammasome that controls the processing of the IL-1 β cytokine precursor pro-IL-1 β into IL-1 β (Petrilli et al., 2007). The reduction of IL-1 β release observed in our studies may be due to the presence of the conserved viral IL-1 β -R mentioned above that is secreted late in the life cycle and binds specifically and with high affinity to mature IL-1 β (Alcami and Smith, 1992; Alcami and Smith, 1996) Spriggs et al., 1992). Increased IL-1 β levels are likely to be immunostimulatory, in particular, the presence of IL-1 β is associated with Th1 immunity induction (Filippi et al., 2003) (Von Stebut et al., 2003), with Fas-ligation-induced maturation of murine DCs (Guo et al., 2003) and with an improvement in memory T-cell responses (Khayyamian et al., 2002). Thus a strategy that is being used to enhance the immunogenicity of MVA is the deletion of viral *IL-1 β -R*, as the specific deletion of this viral gene results in enhanced memory CD8 $^{+}$ T-cell responses against MVA or recombinant antigens upon immunization of mice (Cottingham et al., 2008; Garcia-Arriaza et al., 2010; Santra et al., 2004; Staib et al., 2005).

Another cytokine candidate to enhance immune responses due to the central role it plays in the modulation of the immune system, is IL-12 that is clearly downregulated after infection with MVA. This downregulation is probably secondary to the elevated levels of type 1 IFN, that is known to negatively regulate IL-12 (Cousens et al., 1997). IL-12 production by mature DCs promotes the differentiation of CD4 $^{+}$ T lymphocytes into IFN- γ -producing Th1 cells, as well as the differentiation of CD8 $^{+}$ T lymphocytes into cytotoxic cells (Hamza et al., 2010), moreover, some studies suggest that IL-12 and type 1 IFN could be considered as a signal 3 in the activation and differentiation of CD8 $^{+}$ T-cells. Thus, exploration of strategies to enhance IL-12 levels during MVA infection might be beneficial for immunogenicity, as is the case for classical adjuvants (Curtsinger and Mescher, 2010).

To summarize this section, the knowledge gained from these genetic studies will aid vaccine development groups by identifying candidate genes that could be targeted to specifically activate key innate immune responses and so improve immunogenicity.

2.3. Host gene regulation after NYVAC infection analysed by comparative genomic analysis.

The same concerns for the safety of the attenuated vector and for the effects on human host gene expression of MVA infection also apply in the case of NYVAC. Several cDNA microarray studies have analysed the transcriptional response to NYVAC infection in human cells (5 PFU/cells) (Guerra et al., 2006b) Guerra et al., 2007; Royo et al. 2014). In HeLa cells, NYVAC produces an abortive infection due to the global inhibition in protein synthesis that can be blocked by

reintroduction of the C7L gene to the virus (Najera et al., 2006). The microarray profiling revealed great differences in the patterns of gene expression between WR- MVA-and NYVAC-infected cells. Specifically, analysis of the NYVAC microarray data highlighted the upregulation of genes involved in apoptotic processes, correlating with the known ability of this virus to induce apoptosis, a well-known innate cellular response that limits virus replication. The activation of a key apoptosis-related cysteine protease, caspase-9, demonstrated by cDNA microarrays, was also validated by real-time RT-PCR and Western blotting analyses. The study of Guerra et al., (2006) also pointed out the upregulation of genes involved in the NF- κ B pathway during infection with the attenuated strain, a response also observed in MVA infection. This upregulation of NF- κ B correlated with the expression of other components of the NF- κ B pathway such as *I κ B α* , *IL-6* or *the activating transcription factor 3 (ATF-3)* as assayed by cDNA microarrays, confirming the enhanced NF- κ B activity. ATF-3, whose expression in HeLa cells is regulated by PKR activation, as determined using a VACV that expresses PKR (Guerra et al., 2006a) or a MVA that lacks E3 protein (Ludwig et al., 2005), is known to positively influence innate immunity to pneumococcus infection by enhancing TNF- α , IL-1 β , and IFN- γ expression and modulating bacterial clearance (Nguyen et al., 2014).

The changes in host gene expression after NYVAC infection have also been studied in DCs and macrophages (Guerra et al., 2007; Royo et al. 2014). One of the key conclusions of the first study in DCs was that NYVAC upregulates less genes than MVA during infection, particularly striking being the lower levels of transcription of genes involved in the antiviral immune response and cytokine production. The expression of type-I -IFN related molecules in general is notably higher during MVA than NYVAC infection of DCs. NYVAC infection also induced much lower levels of *TNF- α* , *IL-1* and *IL-6*, probably due to the lower activation of NF- κ B, a key pathway for the expression of inflammatory cytokines such as those mentioned previously. Finally, NYVAC infection of DCs, like infection of HeLa cells, is non-progressive due to the initiation of apoptosis processes, and the upregulation of many apoptosis related genes.

The activation of the NF- κ B pathway and apoptosis that impacts negatively on the progression of NYVAC-infection may actually improve the suitability of this virus as a vaccine vector. NF- κ B activation can stimulate the immune response against recombinant products and facilitate clearance of the vector. Recently it has also been recognised that NF- κ B activation, a process normally inhibited by the NYVAC *A52*, *K7*, and *B15* genes, also controls neutrophil recruitment

to the infection site (Di Pilato et al., 2015). Enhanced neutrophil trafficking to the infection site correlated with increased T-cell responses to the vector-delivered antigens, which may greatly benefit the use of NYVAC as a vaccine vector. Increased apoptosis of infected cells can also improve host immune responses as the antigens expressed by apoptotic cells are efficiently taken-up and cross-presented on MHC-I molecules for enhanced priming of cytolytic T-cells (Barnaba, 2013; den Haan and Bevan, 2001). The importance of apoptosis as an antiviral defence against poxvirus infection is suggested by the finding that a number of poxvirus genes encode proteins that interfere with apoptosis in specific cell types (Shisler and Moss, 2001; Smith et al., 2013; Taylor and Barry, 2006). Some of the genes coding for these proteins critical for the control of apoptosis, such as the serpin homologs encoded by the gene *B14R* or *B13R* are deleted in both MVA and NYVAC (Kettle et al., 1997; Wyatt et al., 1998). Other viral gene products known to block apoptosis include *E3L* (*MVA050L*) (acting through sequestration of dsRNA and prevention of activation of PKR and the 2-5A system) (Chang et al., 1992; Garcia et al., 2007; Rivas et al., 1998) and *FIL* (*MVA029L*), an inhibitor of mitochondrial caspase 9-induced apoptosis (Postigo et al., 2006). Both the *E3L* and *FIL* genes are present in NYVAC and MVA. However another viral gene involved in apoptosis regulation, *NIL* is absent from the NYVAC genome (Kvansakul et al., 2008). Interestingly, however, for both *FIL* and *NIL*, inhibition of innate immune signalling was more important for virus virulence than inhibition of apoptosis (Gerlic et al., 2013; Maluquer de Motes et al., 2011).

Comparison of MVA and NYVAC infection of human DCs reveals specific differences in host gene expression. When cDNA microarray data from DCs infected with MVA and NYVAC were evaluated for virus-vector specific transcriptional differences by removing those genes regulated equally by both viruses from the processed data; 544 genes showed expression pattern differences after poxvirus infection (4.4% of the genes selected), 283 of which had at least two fold-higher transcriptional levels after MVA than after NYVAC infection. Genes including *TNF- α* , *IFN- α* and *IL-12* were increased by fivefold or higher after MVA infection compared to NYVAC infection. These microarray findings indicate that the MVA and NYVAC vectors trigger similar but also distinct regulatory pathways, with MVA infection leading to the upregulation of more host genes than NYVAC.

In the case of macrophages, Royo et al. (2014) confirmed by microarray analyses that genes related to the type I IFN pathway including *IFN- α* , *IFN- β 1*, *IFITM-1* or *ISG15* were upregulated during

NYVAC infection. Antiapoptotic genes such as *BCL2*, *BCL2A1*, *BCL2L1* or the *myeloid cell leukemia 1 (MCL1)* were downregulated, corresponding with the wide range of strategies to counteract apoptosis that poxvirus are known to deploy. Similarly to what was observed in DCs, apoptosis activators like *CASP-8* were upregulated and again this may effectively enhance antigen cross-presentation. As has been described in DCs, NYVAC infection of macrophages also upregulated expression of NF- κ B pathway components, but, differently from DCs interleukins like *IL-6*, *IL-1* or *IL-7* were downregulated. Thus, although NYVAC and MVA are potent activators of the different components of the innate immune system, this potential is less clear in NYVAC, with less upregulation of type I IFN and apoptosis pathways in macrophages. The explanation for the differences observed in gene expression profiling between the attenuated VACV strains is most probably due to the different viral genes deleted during the derivation of each strain. For example, the presence of the viral *A53R* gene in NYVAC genome is probably the reason why TNF- α levels are lower than in MVA-infection. So, these studies may be really useful to improve vector tools, as the differences in host gene expression and levels of immunomodulatory molecules produced during infection in immune cells can be related to identify strategies that might affect the quality of the immune response induced by the different vectors when used as vaccines.

Apart from being useful tools to analyse the gene expression profile after a viral infection, comparative genomic studies serve to detect changes in the behaviour of a virus when a modification is made in its genome, and an ORF is altered damaging a gene responsible of the activation of different pathways. To sum up, we show a model (Figure #3) in which the absence or presence of selected genes in the MVA or NYVAC vector genomes is represented, and more concretely, the resulting activation of different cellular pathways and its effects both from the standpoint of the virus and the target cell.

2.4. Host gene regulation after ALVAC analysed by comparative genomic analysis.

Recently, Teigler et al. (2014) compared the gene expression patterns observed in human peripheral blood mononuclear cells (PBMCs) infected with MVA, NYVAC or ALVAC strains. These studies emphasised the contrast in the innate immune profiles elicited by the different non replicative vectors. Specifically, the infection of human PBMCs with ALVAC elicited higher levels of *IFN- γ* , *IL-1 β* , *IL-6*, and *TNF- α* expression than seen after MVA or NYVAC infection. Furthermore, the phenotype stimulated by ALVAC infection was influenced by several PBMC subsets such as T-cells, monocytes, macrophages and DCs. The depletion of DCs decreased IFN-

$\alpha 2$ levels; depletion of monocytes and macrophages reduced the induction of *IL-1 β* and *TNF- α* and the depletion of T-cells markedly decreased the levels of IFN- γ produced. Similar trends were observed, with lowered levels of elicited *IL1- β* and *TNF- α* , in response to MVA or NYVAC stimulation following depletion of monocytes and macrophages. Interestingly, the impact of DC depletion on IFN- $\alpha 2$ secretion was more modest after MVA or NYVAC stimulation than after ALVAC stimulation. Altogether, these results indicate that multiple PBMCs subsets likely contribute to the overall cytokine production elicited by the ALVAC, MVA, and NYVAC vectors (Teigler et al., 2014).

These studies also showed that vaccination with ALVAC elicited an innate immune response qualitatively and quantitatively different from that elicited by MVA and NYVAC, both *in vivo* in rhesus monkeys and *in vitro* in human PBMCs, characterized by a higher induction of proinflammatory cytokines and IFN-related antiviral molecules on day 1 following immunization (Teigler et al., 2014). The stimulatory phenotypes observed following priming with ALVAC, MVA, or NYVAC were all reduced when these poxvirus vectors were used as a boost. These data suggest potentially important biological differences in innate stimulatory phenotypes between these three clinically relevant poxvirus vectors that are probably related to their different arsenals of immune regulatory genes. Poxviruses possess a wide array of proteins that serve to block host antiviral immune responses (Perdiguero and Esteban, 2009) ALVAC differs significantly from MVA and NYVAC in terms of genome size and in the number of ORFs (McFadden, 2005). As we said previously, many of ALVAC's ORFs may not be functional in mammalian cells, as evidenced by the inability of avipoxviruses to replicate in mammalian cells, and this characteristic may underlie its improved safety profile as a vaccine vector (Meyer et al., 1991; Taylor et al., 1988).

In all the experiments discussed above high multiplicity of infection is used ensuring complete infection, but also is very important to analyze the changes in gene expression where only a fraction of the cell population is infected. For instance, this is usually the case of *in vivo* experiments, in this sense, a pioneer poxvirus genomics experiments performed in blood samples of variola virus infected-macaques showed that the gene expression patterns of specific pathways including the IFN response, cell proliferation and immunoglobulin production was clearly viral dose-dependent, indicating a viral modulation of the host immune response (Rubins et al., 2004).

2.5. Microarray analysis performed for HIV poxvirus derived vaccines.

As discussed previously, the use of microarrays has enhanced our understanding of the host response to infection and provided information that can help elucidate the innate and adaptive immune mechanisms essential for protection against pathogens, as well as the virulence mechanisms deployed by the pathogen. This last part of the review will evaluate the impact of these technologies on the ability to assess the host response and how this has been applied to aid the development of HIV vaccines. Profiling of the immune responses triggered by poxvirus vaccine vectors is critical not only for optimal design of vaccine vectors but also for anticipating potential harmful interactions between naturally acquired or vaccine-induced immune responses against the vaccine used.

MVA is a leading vaccine candidate for delivery of HIV genes with efficient induction of T-cell mediated immune responses (Gomez et al., 2011; Guerra et al., 2010). As described previously, MVA is an effective vector able to activate innate immune responses that might enhance the vector's efficacy when used as a vaccine. Several MVA-based vectors have been developed as promising vaccines against different pathogens but especial effort has been made against HIV-1. One example of these is the vector MVA-B, a modified VACV expressing the Env-Gag-Pol-Nef-encoding genes of HIV clade B (Gomez et al., 2007). To study the effect of MVA-driven expression of HIV antigens on immature DCs, DNA microarray studies were useful again (Guerra et al., 2010). DCs extracted from healthy donors were infected with either MVA or MVA-B (5 PFU/cell and 6hpi) and then analysed using cDNA microarrays. Apart from the previously described upregulation of chemokine genes during the infection of human DCs with MVA (Guerra et al., 2007), HIV antigen expression by MVA-B seemed to clearly enhance the activation of other chemokine genes including *CCL3*, *CCL4*, *CCL20*, *CXCL10*, *CXCL13*, and *CCR8*. MVA-B-infected cells have even higher levels of induction of genes implicated in the immune response, such as *IFN-β*, *TNF-α*, *IFN-α*, *JAK1*, *ISG15*, and several cytokines like *IL-1*, *IL-8* and *IL-6* than in cells infected with only MVA. The effect of the upregulation of these genes could further increase the immunogenicity of MVA-B compared to the parental MVA virus. For example, increased IFN has an important effect in HIV patients (Sandler et al., 2014). These multiple differences in the profiles of host genes between MVA and MVA-B- infected cells, and the specifically enhanced expression of genes involved in different immunomodulation pathways after MVA-B infection suggested a key role of the expression of HIV antigens in these responses. Moreover, MVA-B vaccination may favour the creation of a microenvironment where antigen-presenting cells and

activated T-cells interact to help expand CD4⁺ and CD8⁺ T-cell populations, one of the main requirements for a HIV candidate vaccine. These experiments also identified the MHC class I polypeptide-related sequence (*MICA*) as a gene specifically upregulated after MVA-B infection. *MICA* is a ligand for the activating Natural Killer (NK) receptor G 2D (NKG2D), that stimulates the activation of naïve T-cells and NK cells (Bauer et al., 1999; Maasho et al., 2005; Verneris et al., 2004). Importantly, the *MICA* induced on the surface of MVA-B-infected DCs was functionally active in that these cells were more potent stimulators of the activation of primary human NK cells. Thus, the induction of *MICA* on MVA-B-infected DCs may actually enhance the generation of an adaptive immune response to the vaccine antigens.

This pioneering work allowed identification of cellular genes whose expression levels are markedly regulated following the expression of HIV antigens from the MVA-B vector and that might act as regulators of immune responses to HIV antigens.

Another MVA-based vector that shows some promise as an HIV vaccine is MVA-C, an attenuated strain expressing the HIV-1 antigens Env/Gag-Pol-Nef of HIV-1 of clade C, the predominant subtype virus in Africa and Asia, the most affected areas in the world. Using gene microarray analysis, the effects of MVA-C-infection on the patterns of gene expression of human myeloid (mDCs) and plasmacytoid (pDCs) DCs have been studied by Gómez et al. 2012. These data demonstrate that MVA-C induces the expression, in both cell types, of a large number of biologically significant genes, involved in the regulation of innate and adaptive immune responses (Gomez et al., 2012a). MVA-C infection of mDCs triggered significantly increased expression of *IFN-β* and IFN-induced genes (*IFIT2*, *IFIT3*, *OASL*, *GBP4*), pro-inflammatory molecules (*CXCL9*, *CXCL10*, *TNF-α*) and antiviral genes such as *Zinc Finger CCCH-Type*, *Antiviral 1* (*ZC3HAV1*) that has been shown to have a potent anti-retroviral activity (Zhu et al., 2011). Similarly, in pDCs there was a significant upregulation of IFN genes (*IFN-β*, *IFN-α2*, *IFNα-16*), IFN-induced genes (*IFIT3*) and anti-retroviral genes such as *Tripartite motif-containing protein 5* (*TRIM5*). Moreover, upregulation of genes related to specific immune functions like DCs maturation and activation (CD40, CD80) or T-cell and B-cell activity (*IL-6*, *IL-12*, *IL-28*) was also detected. As well as this activation of expression of highly relevant immunostimulatory molecules, the authors also demonstrated the capacity of MVA-C to express high levels of the HIV antigens that it contains, as well as the induction of DC maturation and cross-presentation; proliferation of CD8 T-cells, enhanced production of antibodies to the Env protein and the generation of an HIV-

1-specific memory T-cell response. In aggregate these results suggest that MVA-C could be an excellent immunogen, with the capacity to activate both humoral and cellular components of the immune response, and therefore, a very promising candidate to be tested as an HIV vaccine in future clinical trials.

Subsequently, several studies have been published related to this issue, but using deletion mutants. The deletion or reinsertion of specific viral genes is a common strategy used in order to improve the ability of the vector to stimulate a host immune response. Using this approach a virus that lacks the viral *N2L* gene, involved in the blockade of IRF3 to inhibit IFN signalling (Ferguson et al., 2013), has been generated to study this effect in the MVA context. The study of host gene regulation by the MVA-BΔN2L mutant virus in human THP-1 cells demonstrated the induction of *IFN-β*, *TNF-α*, *MIP-1α* and *RANTES* genes (Garcia-Arriaza et al., 2014). *RIG-I* and *MDA-5* were also upregulated, validating previous microarray data. Further detailed studies showed increased innate stimulation by these vectors following further modification of their repertoire of immune regulatory gene, highlighting the role of the immune gene repertoire of poxvirus vectors in their innate stimulatory phenotypes.

In the case of NYVAC-based vectors, Quakkelaar et al. (2011) and Kibler et al. (2011) used microarray analysis to compare how different NYVAC viruses used as HIV-1 vaccine candidates triggered the innate immune response during DCs infection. Quakkelaar et al. (2011) studied a virus lacking the *B19R* viral gene that encodes a viral IFN receptor. This virus termed NYVAC-C-ΔB19R triggered, as expected, an enhanced expression of genes related to IFN-induced pathways (*NF-κB1*, *IFN-α* or *TRAFD*), IFN-regulated transcriptional factors (*IRF1*, *IRF2*, *IRF7*) and proinflammatory cytokines. On the other hand, the reinsertion into NYVAC of two genes involved in host range restriction, *KIL* and *C7L*, in order to restore replication competence, led to the generation of the vector named NYVAC-C-KC by the same author. Infection of DCs with NYVAC-C-KC resulted in enhanced expression of antigen processing and presentation related genes and B-cell function related genes, but no induction of IFN-induced or proinflammatory pathway related genes was observed. Another NYVAC derivative where both genetic modifications had been introduced, stimulated expression of both IFN-related genes and those involved in antigen processing and presentation. These results identify a mechanism for poxvirus-induced immune response and alternatives for vaccine vector design.

To summarize section 2, we show in Table 2 the principal genes regulated by the different VACV-based vectors mentioned along this review during the infection of different human cells, being classified in functional categories.

2.6. New advances using deep sequencing technologies.

The recently developed RNA sequencing (RNA-seq) technique provides a conceptually novel approach to the study of transcriptomes allowing the host and pathogen transcriptomes to be analyzed simultaneously (Westermann et al., 2012). In poxviruses, RNA-seq has been used to revisit temporal trends in gene expression to identify interactions between the host and poxviral transcriptome and to map transcription start and stop sites precisely (Yang et al., 2010; Yang et al., 2011a; Yang et al., 2011b). Deep sequencing of VACV and host cell transcriptomes showed that, although overall amounts of mRNA were comparable in infected and uninfected cells, the proportion attributable to the virus was shown to change, specifically in HeLa infected cells at 4 h post-infection 25 to 55% of poly(A)⁺ RNA sequences were viral (Yang et al., 2010; Yang et al., 2011a; Yang et al., 2011b). In A594 cells infected with rabbitpox virus, a decay in overall cellular mRNA levels started at 2.5 hpi, and, by 5 hpi, mRNA levels were drastically lower for the majority of cellular genes (Brum et al., 2003).

Dual RNA-seq has also been used to evaluate the gene expression changes following smallpox vaccination. PBMCs taken from Dryvax vaccinated individuals were either stimulated with or without live Vaccinia virus for 8 hours (Kennedy et al., 2013). Results showed detection of all annotated Vaccinia genes, with those genes classified as “early” in the viral life cycle expressed at significantly higher levels. On the host side, numerous innate genes and pathways were activated upon vaccinia infection, but a number of chemokines, cytokines, interferons, and macrophage-associated genes were downregulated whilst there was an upregulation of expression of histones, IFN β , IFN γ , and heat shock proteins.

3. Conclusion

“Omic” technologies have greatly aided our ability to study the host response during the course of an infection and have provided important insights into the biology of host-virus interactions. Our results indicate that the innate immune profiles elicited by the leading poxvirus vaccine vectors are different, suggesting potentially important biological differences. The extent to which these properties are advantageous to vaccines, however, remains to be determined. Taken together, these data suggest that vectors from the same family can differ markedly in their biological and

innate stimulatory properties, which may potentially impact the resultant adaptive immune responses and protective efficacy of vector-based vaccines.

Figure legends.

Table 1. Representative human genes specifically regulated in macrophages MVA-infected compared to CVA-infected according to predicted biological function. Gene symbol, gene name, fold change in log and p- value are indicated.

Table 2. Differentially regulated genes in diverse human cells infected with different poxvirus vectors. The virus analyzed, the cellular type, the gene description, the gene symbol and the reference is indicated.

Figure #1. Ingenuity pathway analysis showing selected canonical pathways significantly modulated in macrophages infected with MVA compared to those infected with CVA ($p < 0.05$).

Figure #2. Molecular interaction networks focussed on the regulation of IFN- γ (A) and TNF- α (B) built using ingenuity program. Solid lines show direct molecular interactions, dashed lines show indirect molecular interactions. Yellow molecules are those upregulated MVA-infected relative to CVA-infected macrophages, while blue molecules are downregulated.

Figure #3. Representative model indicating the most regulated signalling pathways after MVA or NYVAC infection and the known viral genes that interfere with them. The name of the viral gene that is inactive in MVA is surrounded by a circle with dashed line; the name of the viral gene that is inactive in NYVAC is surrounded by a circle with solid line; and the name of the viral gene that is inactive in MVA and NYVAC is surrounded by a square. The name of the cellular gene that is upregulated after MVA infection is surrounded by a circle with dashed line; the name of the cellular gene that is upregulated after NYVAC infection is surrounded by a circle with solid line; and the name of the cellular gene that is upregulated by MVA and NYVAC is surrounded by a square. Based on (Smith et al., 2013)

Supplementary information legends

Supplementary Table 1. Differentially regulated genes in macrophages infected with different poxvirus vectors. Comparison of gene expression profiling between MVA- and CVA-infected macrophages. Representative human genes specifically regulated by each vector according to predicted biological function. Gene symbol, gene name, fold change in log and p-value are indicated.

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