The early phase of the vaccinia virus replication induces cytokine gene expression in macrophages

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Dedicated to my lovely mother and sister, my father and my grandparents for their love and support….

“The roots of education are bitter, but the fruit is sweet”
Aristotles.

Contents

1. INTRODUCTION

Vaccinia virus (VACV) belongs to the Poxviridae, which comprise a large family of complex DNA viruses. VACV is the prototypic and best characterized member of the Orthopoxvirus genus (Condit et al, 2006). VACV was the live vaccine used in the vaccination program coordinated by the World Health Organization that lead to the eradication of human smallpox in 1977 being the only extinct human infectious disease worldwide (Fenner, 1980). Despite the importance of VACV strains as vaccines against variola virus, theirs use was associated with a high incidence of adverse events after primary vaccination. Therefore, highly attenuated strains of VACV were developed to overcome those risks. Modified Vaccinia virus Ankara (MVA) was derived from Chorioallantois vaccinia virus Ankara (CVA) by more than 570 passages in chicken embryofibroblasts cells (CEF) (Mayr & Munz, 1964). MVA became replication deficient in almost all mammalian cells, however maintaining good immunogenicity. The diminished virulence in mammalian hosts further improves the safety of MVA as a vector. Despite the considerable loss of genetic material, MVA is still able to efficiently express recombinant genes in infected cells (Mayr et al, 1978). Thus, MVA is a promising candidate vector for antigen delivery against different infectious diseases in animals and humans, including influenza, tuberculosis and HIV (Gilbert, 2013). Additionally the importance of MVA-based smallpox vaccine has been renewed due to the risk of accidental or intentional release of variola virus (Reardon, 2014). MVA no longer encodes many of the immunomodulatory factors produced by conventional VACV strains, and as a consequence this leads to rapid local immune responses (McFadden, 2005). Yet, how the viruses activate the innate immune system remains incompletely understood. For these reasons, yet it is desirable to elucidate the molecular immune mechanisms activated upon MVA

infection. This better understanding should help to further improve the development and rational use of new MVA-based vaccines.

2. LITERATURE REVIEW

2.1 Poxviruses

2.1.1 Taxonomy of Poxviruses

Viruses of the *Poxviridae* family are divided into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae*, based on vertebrate and insect host range respectively. The *Chordopoxvirinae* consists of ten genera: *Avipoxvirus*, *Cervidpoxvirus, Crocodylipoxvirus*, *Capripoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus and Yatapoxvirus.* Four genera contain species that infect humans: *Molluscipoxvirus, Orthopoxvirus, Parapoxvirus* and *Yatapoxvirus* (Baxby & Bennett, 1997). The members of a same genus possess similar morphology and are genetically related (Condit et al, 2006).

2.1.2 Morphology

Poxvirus particles are exceptionally large; its size is around 250 nm in diameter and 360 nm in length, characterized by having a round brick-shape (Fig. 2.1). There are two different infectious particles: internal mature virus (IMV) and extracellular enveloped virus (EEV), they are structurally similar, however EEV carry an additional outer lipid membrane containing proteins absent from IMV(Fig. 2.1) (Locker et al, 2000).

Poxviruses virions contain a large single linear double-stranded deoxyribonucleic acid (dsDNA) genome of 130-300 kilobase pairs (kb), with termini that form covalently closed hairpin loops. *Chordopoxvirinae* encode between 136 and 260 open reading frames (ORF) approximately (Schramm & Locker, 2005). Essential genes are located in the central region of the genome and are highly conserved among poxviruses, while end terminal genes are non-essential for replication, more diverse and usually related to immune subversion or host range restriction (Werden

et al, 2008). The viral genome is packaged into a biconcave core flanked on two sides by structures called lateral bodies, that are composed of viral proteins (Cyrklaff et al, 2005). The poxvirus virions also include the multisubunit viral DNA dependent ribonucleic acid (RNA) polymerase, the mRNA capping enzyme and other factors needed for the early stages of transcription (Senkevich et al, 2005) .

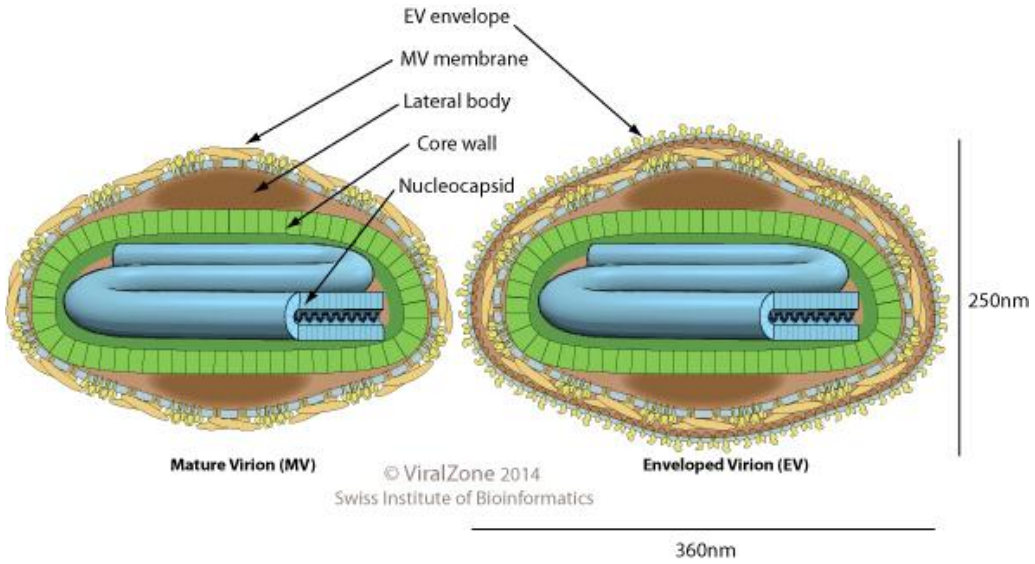

Fig.2.1 Poxvirus morphology (ViralZone.ExPASy.SIB, with permission).

2.1.3 Replication cycle

The binding of the virion is determined by viral proteins and by glycosaminoglycans (GAGs) on the surface of the target cell (Lin et al, 2000). The precise mechanism of poxvirus entry is not fully understood, but it has been proposed that a macropinocytosis process is involved for the release of the core into the cytoplasm (Fig. 2.2) (Mercer & Helenius, 2008). Within this core around 100 mRNAs are transcribed, facilitated by the viral 'early' transcription machinery that is packaged into the virion (Broyles, 2003). The early mRNAs synthetized inside the genome-containing core are then extruded to the cytoplasm for translation. Poxviral proteins translated from these early mRNAs serve to replicate the virus DNA, modify the host cell to the viral advantage and to block the host innate immune response (Joklik,

1964; Sarov & Joklik, 1972; Smith et al, 1997). For degradation of the core and uncoating of the DNA early viral proteins have to be expressed, one or more of the early proteins are responsible for making the ubiquitinated core proteins accessible to the proteasome and then degraded (Mercer et al, 2012).

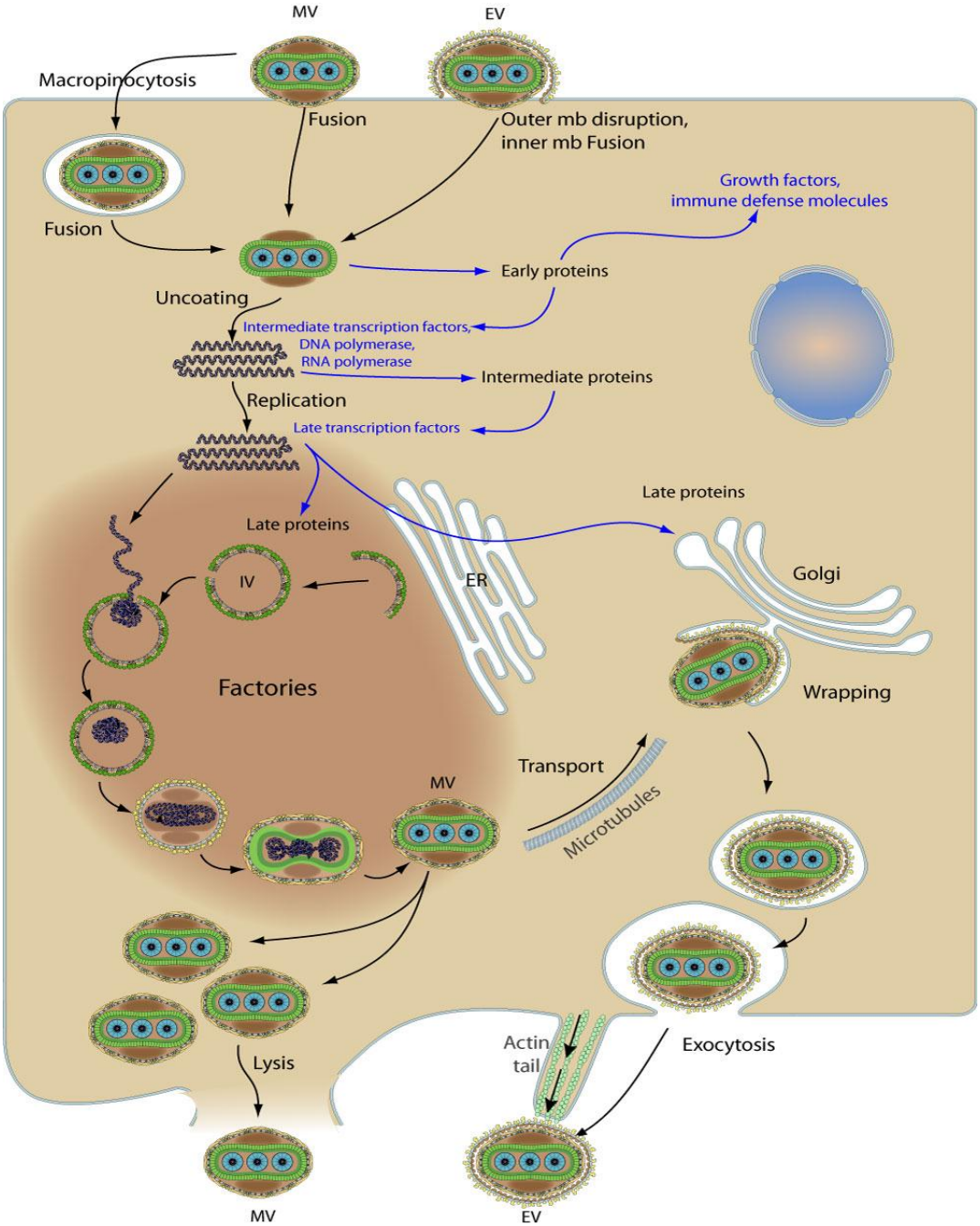

Fig.2.2 Cellular cycle of poxvirus infection (ViralZone.ExPASy.SIB, with permission).

After DNA replication has started, the transcription of intermediate genes commences, which are fewer in number and encode mostly regulatory proteins that

induce the transcription of late genes. The late genes encode most of the virus proteins for building new virus particles and enzymes that are packaged into virions to initiate transcription in the next viral cycle (Assarsson et al, 2008). Unlike early transcription the intermediate and late transcription stages require the interaction with host-derived transcription factors that contribute to the efficiency of these later waves of viral gene expression (Katsafanas & Moss, 2004; Rosales et al, 1994; Sanz & Moss, 1998).

Accumulation of late viral gene products drives the progressive morphogenesis and assembly of infectious virus particles, initially as IMV virions, in structures known as viral factories (Sodeik & Krijnse-Locker, 2002). IMV assemble and migrate via microtubule mediated trafficking and wrap with Golgi-derived membranes to form internal enveloped virus (Fig. 2.2) (IEV) (Sodeik & Krijnse-Locker, 2002). IEV fuses with the cell surface membrane to form cell-associated enveloped virus (CEV), which is either extruded from the cell by actin-tail polymerization or released to form free extracellular enveloped virus EEV (Fig. 2.2) (Roberts & Smith, 2008).

2.1.4 Poxviral diseases in humans

Smallpox was a devastating human infectious disease caused by variola virus (genus Orthopoxvirus), during the last millennia it has been estimated that this disease was responsible for 10% of mortalities worldwide (Mercer, 1985). Smallpox made no distinctions; it affected all ages and socioeconomic classes. The main symptoms included high fever, chills or rigors, cephalagia, characteristic dorsal-lumbar pain, myalgias, prostration, nausea and vomiting. After 4 days, the fever receded and a rash appeared around the eyes, on the face and subsequently would cover the whole body. Other clinical forms of the disease existed, persons with fulminating smallpox (purpura variolosa) had mucocutaneous hemorrhages that preceded the appearance of the typical skin lesions (Barquet & Domingo, 1997). In malignant smallpox, the

rash had a slow evolution characterized by pseudocropping, subconjunctival hemorrhages, and death when lesions on the limbs and face were confluent (Barquet & Domingo, 1997). The mortality rate associated with smallpox varied between 20% and 60% and most survivors were left with disfiguring scars (Mercer, 1985).

In 1796 Edward Jenner performed an experiment that laid the groundwork for the eradication of smallpox and by promoting vaccination using a cowpox infection (Baxby, 1996). Two centuries later in May 1980 and after a global vaccination campaign the World Health Organization declared that smallpox had been eradicated (Fenner, 1980). To date it is the only human infectious disease to be completely extinct. However samples stocks of variola still exists in the United States at the Centers for Disease Control and Prevention and by Russia at the State Research Centre of Virology and Biotechnology (Li et al, 2007). Although, recently forgotten vials containing smallpox were founded in the National Institutes of Health (NIH) campus in Bethesda, Maryland, and many experts believe that numerous stocks still exist around the world (Reardon, 2014). Thus, the probability that stolen variola cultures may be used as bioterrorist weapons remains an important subject of international concern.

Recently, concern has been raised about potential outbreaks of infectious diseases that clinically mimic smallpox, particularly if it is caused by a novel or emerging agent. That threat of novel zoonotic infections was confirmed in 2003, when an outbreak of a pox-like illness in people occurred in the central USA. This outbreak was attributed to the monkeypox virus (MPV), a rare zoonosis that can cause illness clinically indistinguishable from smallpox (Di Giulio & Eckburg, 2004; Reed et al, 2004). The natural animal reservoir of the MPV is unknown but it is believed that rodents were the possible source of its introduction into the United States (Reed *et al.*, 2004). That outbreak was not particularly aggressive; however it is better to be prepared for

future threats where a VACV vector would be required as a vaccine against potential poxviral zoonotic infections.

2.1.5 Immune evasion

VACV like other poxviruses carry a large genome that encode multiple classes of immunomodulatory proteins that have evolved specifically to inhibit diverse process such as apoptosis, the production of interferons (IFNs), chemokines, inflammatory cytokines, the activity of cytotoxic T lymphocytes (CTLs), natural killers (NK) cells, complement and antibodies (Haga & Bowie, 2005; Seet et al, 2003). Poxvirus immunomodulatory proteins can be divided by function in three distinct strategic classes: virostealth, virotransduction and viromimicry (Johnston & McFadden, 2003). Virostealth is the capacity that some poxviruses possess to mask the visible signals associated with the infection, mostly by downreagulating the antigen recognition or blocking the presentation of viral antigen to immune cells. Major histocompatibility complex class I (MHCI) depletion and cluster of differentiation 4 (CD4) coreceptor downregulation are the main examples of this mechanism (Moss & Shisler, 2001).

Virotransducers are intracellular viral proteins that inhibit innate antiviral pathways, such as apoptosis, proinflammatory cascades or the induction of the antiviral state; several of these poxvirus genes have been hijacked directly from the host immune system (Seet *et al.*, 2003). Virotransducers can also target host signal transduction pathways that influence host range (Johnston & McFadden, 2003). One of the best characterized is VACV E3, a doublestranded RNA (dsRNA)-binding protein that inhibits activation of protein kinase RNA-activated (PKR) and blocks IFN responses by sequestering dsRNA molecules, also affect the IFN-stimulated genes (ISG) gene 2'-5'-oligoadenylate synthetase (OAS) antiviral pathway (Davies et al, 1993). VACV also encode several immunomodulators that interfere with different pattern recognition receptor (PRR) activation pathways, including toll-like receptor (TLRs)

and cytoplasmatic retinoic acid inducible gene (RIG-I)-like receptors (RLRs) (Haga & Bowie, 2005). One of the main signaling factors blocked by numerous poxviral proteins (including N1, B14, K1L and M2) is the nuclear factor κappa-enhancer of activated B cells (NF-κB), a key mediator of inducible transcription in the innate immune system (Mohamed & McFadden, 2009).

Viromimicry includes virokines and viroceptors that are virus-encoded proteins which mimic host cytokines, chemokines and their receptors. These proteins establish a protected microenvironment for the virus by blocking extracellular communication signals. The main extracellular pathways targeted are involved in early inflammatory responses, and include the modulation of IFNs and proinflamatory cytokines as tumor necrosis factor alfa (TNF-α) and interleukin 1 beta (IL-1β) (Bahar et al, 2008; Johnston & McFadden, 2003).

2.2 Modified Vaccinia virus Ankara (MVA)

Modified Vaccinia virus Ankara (MVA) is a highly attenuated VACV strain that is not able to replicate in human and most other mammalian cells. MVA was generated by serial passages of a Turkish smallpox vaccine strain named Chorioallantois vaccinia Ankara (CVA) in chicken embryo fibroblast (CEF) (Mayr & Munz, 1964; Mayr et al, 1978). After more than 500 *in vitro* passages the virus has lost approximately 30 kb of DNA, equivalent to 15% of the parental viral genome (Fig. 2.3) (Mayr et al, 1978; Meyer et al, 1991). The main DNA loss is located in six major deletion sites at both ends of the genome, additionally it accumulated many point mutations and short deletion in other genes (Fig. 2.3) (Antoine et al, 1998). As result MVA lacks the capacity to encode several proteins involved in immune-modulation and host-interaction process (McFadden, 2005; Meyer et al, 1991).

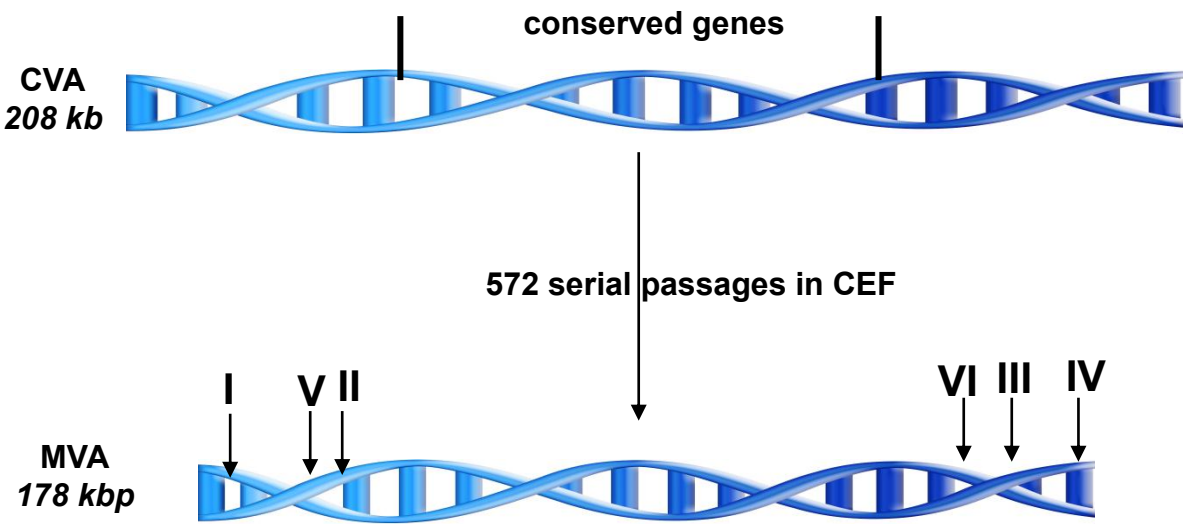

Fig.2.3 Generation of modified vaccinia virus Ankara. (Adapted from Sutter LMU thesis 1990, with permission)

Consequently MVA is replication-deficient in most of mammalian cells (Blanchard et al, 1998; Carroll & Moss, 1997), as viral multiplication is arrested at later stage of the virus cycle after accumulation of immature particles in the cytosol (McFadden, 2005; Sutter & Moss, 1992). Due to its safety and limited replication, MVA was tested as smallpox vaccine by the Bavarian State Vaccine Institute and more than 120 000 individuals were vaccinated without any major adverse events (Stickl et al, 1974). Despite the loss of several genes, MVA has a potent immunogenic capacity compared with other VACV strains. Even though the replication of MVA is abortive in most of mammalian cells, the virus is still able to efficiently express recombinant genes, which makes it a promising candidate vector for antigen delivery against different infectious diseases in animals and humans (Sutter & Moss, 1992).

MVA no longer encodes many of the known poxviral virulence factors, and even though the impaired replication capacity of MVA is well established, the genetic basis for this host range restriction is not fully understood (Johnston and McFadden, 2003).

Two of the classical host range genes of VACV are truncated in MVA: K1L and C12L, while E3L, K3L and C7L are still present (Antoine et al, 1998). The reconstitution of these host range factors only partially rescue the replication capacity of MVA in selected cell lines (Sutter et al, 1994; Wyatt et al, 1998). The lack of the pathogenicity of MVA has been mostly associated with the loss of gene function including the six major deletions in its genome but also the many other mutations present in the MVA genome (Antoine et al, 1998; Meyer et al, 1991). This was nicely illustrated by a study by Meisinger *et al*., attempting to obtain an MVA-like phenotype by introducing the six major deletions in a CVA genome. The results showed that CVA variants with the major deletions were only incompletely attenuated in a murine model and did not reproduce the characteristic host range phenotype of MVA (Meisinger-Henschel et al, 2010). Thus point mutations and/or smaller deletions in gene sequences outside the six major deletions definitively contribute to the properties of MVA attenuation. More studies are required to elucidate the full genetic basis of the safety and immunogenicity of this vector virus. Interestingly inactivation of the E3L gene in the MVA genome resulted in enhanced production of Type I IFNs in CEFs, suggesting that the capacity of MVA to stimulate innate response can be also further improved by reasonable mutagenesis (Hornemann et al, 2003; Ishii et al, 2006) .

Immunization with MVA protects mice against the virulent VACV Western Reserve (WR) infection and also protects them from the lethal challenge with mousepox virus, in a prophylactic and therapeutic approach (Drexler et al, 2003; Paran et al, 2009; Samuelsson et al, 2008; Staib et al, 2006). Additionally Earl *et al.* have shown a high protective capacity of MVA vaccine in non-human primates against monkeypox challenge (Earl et al, 2004; Volz & Sutter, 2013).

Some clinical trials using recombinant MVA for treatment of human immunodeficiency virus (HIV) infection, malaria and other infectious diseases has

been encouraging and confirm their safety (Cosma et al, 2003; Gilbert, 2013; Gilbert et al, 2006). MVA is a very promising viral vector vaccine candidate because it has a high safety profile combined with robust immunostimulatory abilities while its capacity to deliver recombinant antigens is conserved. In the last decades, MVA has been proposed as a vaccine against a possible smallpox-related bioterrorist attack (Belyakov et al, 2003). While there is a major interest in MVA as a vector and as a vaccine, how it activates the innate immune system remains still incompletely understood. It is likely that these innate responses also influence the extent and efficacy of responses by the adaptive immune system. For these reasons it is crucial to better elucidate the cellular and molecular immune mechanisms modulated by MVA, since this should help in the rational development of new MVA-based vaccines.

2.3 Innate immune activation

The host immune response to VACV infection consists of two steps. Firstly innate effectors such macrophages, NK cells and type I IFNs are critical in the early phase. Secondly, adaptive antigen-specific T and B cell response, which are essential for clearance of the virus and establishment of immunity (Haga & Bowie, 2005). Particularly MVA has the ability to activate robust innate immune responses.

2.3.1 Activation of pattern recognition receptors

Viral infections are detected by sensor molecules, which initiate the antiviral response and activate the innate immune response, including the secretion of type I IFNs and proinflamatory cytokines (Yoneyama & Fujita, 2010b). Three main PRRs families have been related with VACV sensing: TLRs, RLRs and DNA sensor molecules (Fig. 2.4) (Peters et al, 2013; Yoneyama & Fujita, 2010b). Each TLR detects distinct pathogen-associated molecular patterns (PAMPs) derived from pathogens, such as

lipoproteins and lipopolysaccharides (LPS) which are sensed by cell surface TLR2 and TLR4 respectively.

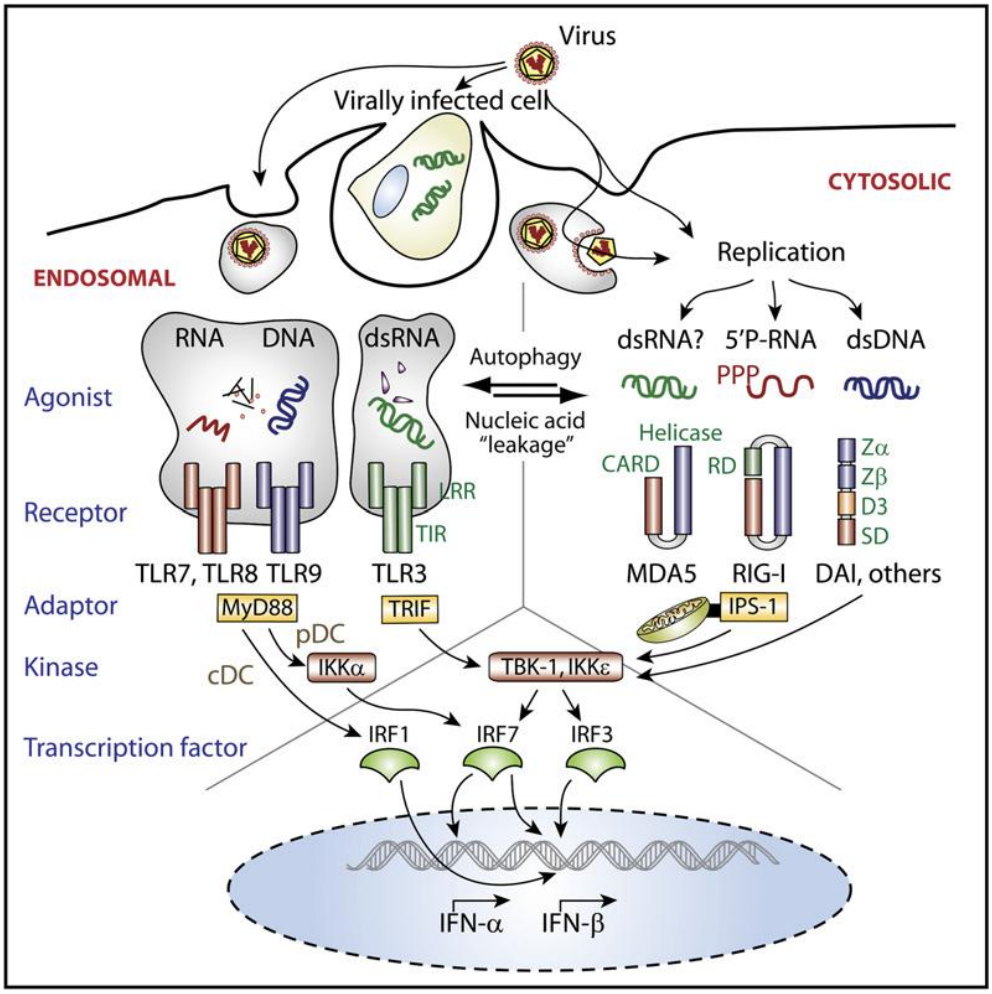

Fig.2.4 Pathways Coupling Virus Recognition to Type I IFN expression.
Leucine-rich repeat (LRR), caspase activating recruitment domain (CARD), RNA-binding domain (Helicase); RD: repressor domain (RD), Z-DNA binding domains (Za, Zb), and signaling domain (SD). (Adapted from Pichlmair *et al.* 2007, with permission)

Viral nucleic acids are detected in endosomal compartments: ds RNA (TLR3), single-stranded (ss) RNA (TLR7 and TLR8) and CpG motifs in DNA (TLR9) (Fig. 2.4) (Kawai & Akira, 2007; Uematsu & Akira, 2004). Upon recognition of respective PAMPs, TLRs recruit specific adaptor molecules such as myeloid differentiation primary response gene 88 (Myd88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) and initiate downstream signaling events that lead to the

secretion of IFNs, inflammatory cytokines and chemokines (Fig. 2.4) (Kawai & Akira, 2011). RLRs are sensor molecules for the detection of viral RNA in the cytoplasm of infected cells, in mammals three family member have been identified RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Fig. 2.4) (Pichlmair & Reis e Sousa, 2007; Yoneyama et al, 2005). Both RIG-I and MDA-5 induce cellular signaling through the adaptor mitochondrial antiviral signaling proteins (MAVS) (Seth et al, 2005). In addition viral RNA activates antiviral effectors including PKR, OAS, adenosine deaminase acting on RNA (ADAR) and RNAse L (Samuel, 2001).

The restrictive replication phenotype of MVA is most likely linked to the cellular signals triggered when the virus enters the host cell, which this vector is not able to evade. The use of microarray studies in infected dendritic cells has shown that MVA induces the expression of several genes involved in innate immune response including RIG-I, MDA-5 and TLRs (Guerra et al, 2007). Additionally a report from Delaloye *et al.* has revealed that in bone marrow derived macrophages *(*BMDM) the TLR2-TLR6-Myd88 and MDA5 pathways are important in modulating the cytokine and chemokine profile produced by MVA infection. Particularly a reduced expression of RIG-I and MDA-5 by shRNAS indicated that sensing of MVA and production of IFN-β and IFN-β-dependent chemokines was controlled by MDA-5 pathway in macrophages (Delaloye et al, 2009). Also Pichlmair *et al.* showed that dsRNA extracted from VACV-infected cells could induce MDA-5-dependent Type I IFN (Pichlmair et al, 2009).

In the last years several studies showed the relevance of TLR2 in sensing VACV infection, however most of the results in vivo are quite inconsistent. Barbalat *et al.* claimed that TLR2 activation by VACV lead to the secretion of IFNs in monocytes (Barbalat et al, 2009), while another study reported significant decrease of

inflammatory cytokines in *Tlr2*$^{-/-}$ mice (Zhu et al, 2007). On the other hand, one study demonstrated that TLR2 has a minor influence in the course of VACV infection in a murine disease model (O'Gorman et al, 2010). Another group has observed that whilst the CD8 T cell response is MyD88-dependent the *Tlr2*$^{-/-}$ mice are still able to produce a robust antiviral immune response (Zhao et al, 2009). These last results are in line with the conclusions of Davies *et al*., who recently have shown that in mice, MyD88 is required for efficient induction of $CD4^+$ T cell and B cell responses, whereas TLR2 is disposable for control of virus replication and induction of adaptive immunity to VACV infection (Davies et al, 2014). Thus, based on these contradictory results, there has been increased concern that many reports involving TLR2 in the sensing of VACV are artifacts of possible contamination, especially as the specific TLR2 ligand so far remains uncharacterized (Davies et al, 2014; Price et al, 2013).

Mice lacking TLR9 are more susceptible to Ectromelia virus (ECTV) infection, the causative agent of mousepox, none the less MVA still protects from disease in *Tlr9*$^{-/-}$ mice (Samuelsson et al, 2008). In contrast, in a mouse model, activation of TLR3 contributed to the pathogenesis of the virulent VACV WR (Hutchens et al, 2008). Mice lacking the adaptors of TLR signaling MyD88 and TRIF showed IFN-α responses that were only slightly reduced compared to those of wild type mice (Waibler et al, 2007).

2.3.2 Type I Interferon

Type I Interferons (IFN-α and IFN-β) are a group of secreted cytokines that constitute the first line of defense against viral infection and can induce direct antiviral effects (Honda et al, 2006; Sen, 2001). These cytokines have various biological activities, including anti-viral, anti-proliferative and immunomodulatory effects. After induction, all type I IFNs bind one common type I IFN receptor (IFNAR) which leads to downstream signaling resulting in the expression of more than 300 ISG (Fig. 2.5)

(Honda et al, 2006; Samuel, 2001). The ISG are involved in eliminating viral components from infected cells, inducing apoptosis of infected cells and conferre resistance to viral infection on uninfected cells (de Veer et al, 2001). Most of the ISG genes are antiviral effectors including the IFN-induced PKR and OAS (Fig. 2.5), which are then activated by dsRNA produced during virus infection (Sen & Sarkar, 2007).

Nevertheless the effects of IFNs are not limited to induction of ISG, they also have a great impact on the systemic immunity. Indeed, it has been reported that IFNs are involved in the maturation of DCs, including the cross-presentation of viral antigens for CD8+ T cell and the activation of NK cells (Burshtyn, 2013; Stetson & Medzhitov, 2006). IFN signaling in poxvirus infection has also been proposed to play a role in the generation of a virus specific CTL response (Kolumam et al, 2005). IFNs have been implicated in the induction of chemokine expression in bone marrow macrophages during murine cytomegalovirus (MCMV) infection (Crane et al, 2009). Mice deficient in IFN receptors are abnormally susceptible to VACV showing the relevance of IFNs in controlling VACV infection (van den Broek et al, 1995) even an IFN therapy protects the mice from the lethal challenge with VACV WR infection (Liu et al, 2004).

VACV like many poxviruses evades this response using various strategies such as the direct suppression of IFN induction or blocking the IFN signaling pathways (Fig. 2.5) (Perdiguero & Esteban, 2009; Smith et al, 2013). VACV encodes soluble viroceptors like B8 that block the binding of IFNs to their cell surface receptors (Jackson et al, 2005). VACV E3L, K3L, C7L, K1L and VH1 proteins block distinct intracellular pathways upstream and downstream of IFN activation (Fig. 2.4) (Perdiguero & Esteban, 2009; Seet et al, 2003; Smith et al, 2013).

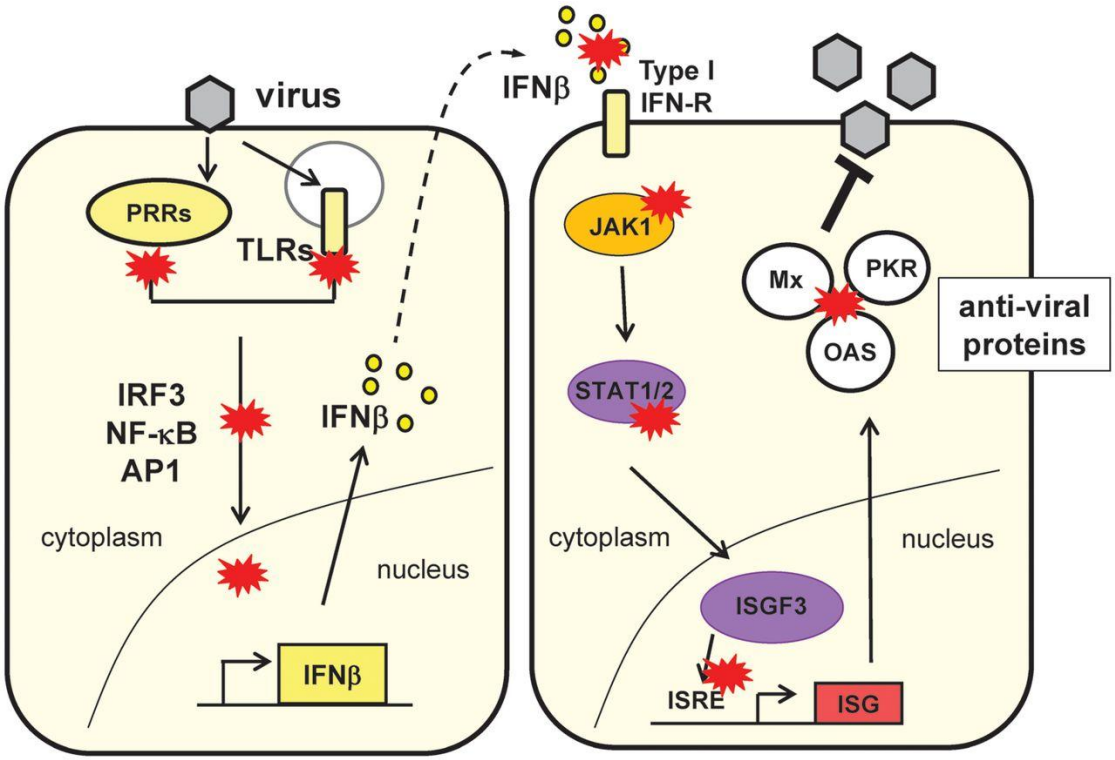

Fig.2.5 IFN signalling and its antagonism by VACV. The positions at which viral proteins can inhibit the production or action of IFN are shown by red stars, and many of these are illustrated by VACV proteins. (Adapted from Smith *et al* 2013, with permission).

Since MVA is highly attenuated, it is not a surprise that the virus has lost several proteins that impair IFNs functionality (Antoine et al, 1998). Therefore MVA has the capability to induce higher levels of IFNs than other VACV strains (Buttner et al, 1995; Waibler et al, 2007), a rapid local immune response and IFN-dependent expansion of virus specific CD8+ T cells (Frenz et al, 2010). However MVA still contains some of these genes like E3L and C7L, indicating that it is possible to further increase its immunostimulatory capacity (Antoine et al, 1998).

2.3.3 Chemokines

Chemokines are a superfamily of small secreted proteins that are classified into four subfamilies denominated C, CC, CXC, and CX3, depending of the arrangements of disulphilde bridges between cysteine residues near their amino terminus (Zlotnik &

Yoshie, 2000). Chemokines are chemoatractant molecules that have a major role in the inflammatory response to pathogen infections (Alcami, 2003). They modulate the migration of leukocytes and up-regulate the expression of leukocytes adhesion molecules. Furthermore, chemokines play a critical role in modulating the innate and adaptive host responses to viral infections (Fauci, 1996; Mahalingam et al, 1999).

Many poxviruses including VACV express proteins that interfere with chemokine functions (Alcami et al, 1998). B7 is one of these proteins, which is not functional in MVA (Alejo et al, 2006). Nevertheless, some other genes such as A41L and B15R are still present in the genome of MVA, which when deleted induce a stronger CD8+ T cell response and conferre better protection to subsequent challenges with virulent VACV (Clark et al, 2006; Staib et al, 2005).

Lehmann *et al.* showed that infection of human monocytic cells with MVA induces a strong chemokine response, with a pronounced upregulation of CC and CXC chemokines. Further antibody inhibition studies *in vitro* demonstrated that the chemokine (C-C motif) ligand 2 (CCL2) induces chemotaxis in the human monocytic cell line THP-1. The importance of CCL2 was corroborated in mice, since it was shown that MVA and no other VACV strains triggers immigration of monocytes, neutrophils and CD4+ lymphocytes into the lung after intranasal infection, correlating with the strong expression of CCL2. Also, using CCL2-deficient mice it was demonstrated that CCL2 plays a key role in the early respiratory migration of leukocytes triggered by MVA (Lehmann et al, 2009). CCL2 is a key factor in the initiation of inflammation and recruitment of monocytes, memory T cells and dendritic cells to the site of infection (Yadav et al, 2010). Usually the CCL2 expression is regulated at the transcriptional level by stimulatory cytokines like TNF-α, acting as a key mediator the NF-κB in the induction of CCL2 expression (Sung et al, 2002). Some studies have reported that IFNAR stimulation lead to the induction of CCL2

expression (Conrady et al, 2013). In fact systemic release of IFN during MCMV infection drives expression of CCL2 in bone marrow cells promoting monocyte migration to the site of infection (Crane et al, 2009).

2.3.4 Macrophages

Cells of the monocyte/macrophage lineage have an important function as antigen presenting cells (APC) for the activation of T cells and in virus-host interactions. These cells play a key role in poxviral immunity. *In vitro* assays have shown that VACV preferentially infects monocytic cells (Sanchez-Puig et al, 2004) and induces apoptosis in a murine macrophage cell line (Humlova et al, 2002). In mouse peritoneal macrophages, the VACV replication cycle was found to be abortive with only early viral protein expressed, the absence of viral DNA synthesis and late proteins prevents assembly of progeny virions (Natuk & Holowczak, 1985). In contrast, in activated rabbit peritoneal macrophages the virus replication cycle is blocked at a later stage after DNA synthesis (Buchmeier et al, 1979). Curiously VACV replicates in macrophages from naive but not immune animals (Avila et al, 1972), with the same result obtained *in vivo* (McLaren et al, 1976). Mice depleted of macrophages are unable to control VACV infections due to impaired virus clearance and antiviral response (Karupiah et al, 1996). In fact, monocytic cells appear to have an important role in limiting virus replication and dissemination (Chapes & Tompkins, 1979). Additionally, murine alveolar macrophages limit the spread of VACV WR, and depletion of these cells increases virus replication and dissemination (Rivera et al, 2007). In a mouse model of intranasal infection, Lehmann *et al.* demonstrated that MVA but no other VACVs strains triggered the immigration of leukocytes into the lung (Lehmann et al, 2009). This early immigration of leukocytes has been correlated with the ability of MVA to confer a short-term immunization more efficiently than other VACV strains, and thus is able to protect mice 48 h before a lethal challenge with the

virulent VACV WR (Staib et al, 2006). Also, postexposure immunization of MVA protects the mice against lethal challenge with ECTV (Paran et al, 2009) .

Besides the direct role played by monocytic cells in controlling poxvirus infection and in the primary innate immune response, there is evidence that these cells may also play a critical role in the development and execution of the adaptive immune response by augmenting the activation of CTLs (Karupiah et al, 1996; Narni-Mancinelli et al, 2011). Therefore monocytes and macrophages are key players in modulating the adaptive response and in the induction of a long term poxviral immunity.

2.4 O-GlcNAcylation

O-linked beta-N-acetylglucosamine (O-GlcNAc) modification of nuclear and cytoplasmatic proteins is important for many cellular processes like protein expression, degradation and trafficking (Hart et al, 2007). O-GlcNAc modification of proteins is catalyzed only by one enzyme called O-linked N-acetylgucosaminyltransferase (OGT) and is removed by the enzyme beta-N-acetylglucosaminidase (Hart et al, 1989). OGT is ubiquitously expressed with high transcript levels in macrophages (Hanover et al, 2010). O-GlcNAcylation is one of the most common posttranslational modifications and together with phosphorylation plays a reciprocal role in regulation of protein function by competing for modification of the same serine or threonine residues (Copeland et al, 2008; Slawson & Hart, 2003). Many transcription factors have been shown to be modified by O-linked GlcNAc modification, which can influence their transcriptional activity, DNA binding, localization, stability and interaction with other co-factors (Ozcan et al, 2010). Some of these transcription factors include NF-κB, nuclear factor of activated T-cells (NFAT), tumor protein 53 (p53), specificity protein 1 (Sp1) and others. In fact over

25% of the O-GlcNAcylated proteins are involved in transcriptional regulation (Ozcan et al, 2010).

Cells of the immune system undergo large changes in their metabolic rates when they are activated by danger signals. Some of the cells enter a phase of extensive and rapid cell proliferation, altering the level of cellular glucose concentration which is though to strongly affect the levels of O-GlcNAc-modified proteins (Golks & Guerini, 2008). Initial reports by Kearse *et al.* showed that treatment with phorbol 12-myristiate 13-acetate (PMA) lead to an increase in nuclear and cytoplasmatic O-GlcNacylated proteins in lymphocytes (Kearse & Hart, 1991). More recent work has reported that O-GlcNAcylation of the transcription factors NF-κB and NFAT is needed for productive T-cell activation in a T-cell receptor-dependent manner (Golks et al, 2007). Similar effects for NFAT and NF-κB were observed during B-cell receptor (BCR)-dependent activation of B cells (Golks et al, 2007). The requirement of the active OGT enzyme for productive T cell activation was consistent with the reduction of CD4+ T cells observed in transgenic mice carrying an inactivating T-cell deletion in the OGT gene (O'Donnell et al, 2004). In neutrophils, chemokine stimulation lead to an increase in O-GlcNAcylated proteins (Kneass & Marchase, 2004) and this O-GlcNAc modification affects their motility and activation (Kneass & Marchase, 2005). Additionally, it was reported that in a monocytic cell line the expression of macrophage Inflammatory proteins 1-α (MIP-1α) and MIP-1β was regulated by the O-GlcNAcylation of nuclear proteins (Chikanishi et al, 2010).

Although there are several viral proteins that are subject to O-GlcNAc modification (Hart et al, 2007; Love & Hanover, 2005), the relevance of this posttranslational modification for host-pathogen interactions has been not extensively studied. Perhaps one of the few examples is the report from Jochman *et al.*, where they

demonstrated that the O-GlcNAcylation of the transcription factor Sp1 inhibits the activity of HIV-1 LTR promoter (Jochmann et al, 2009).

2.5 Objectives

MVA is gaining increased importance as a candidate to be used as an attenuated live vaccine against various infectious diseases in humans and animals. Therefore, it is necessary to gain a better understanding of the mechanisms of MVA-induced immunity. This study aims to (i) Examine which viral components are important for MVA-induced cytokine expression in infected monocytes and macrophages. (ii) Test whether IFNAR modulates CCL2 production in MVA-infected BMDM. (iii) Determine whether O-GlcNAc modification modulates CCL2 expression in MVA-infected monocytes.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Media and Cell culture

Reagent	Supplier
RPMI (Endotoxin free)	Sigma
DMEM (Endotoxin free)	Biochrom
Fetal Calf Serum (FCS) (VLE)	Biochrom
PBS (Endotoxin free)	Biochrom
Trypsin Solution	Sigma
Penicillin/streptomycin	Sigma
Adenosine N1-oxide (ANO)	SIA MolPort (Lithuania)
Cytosine arabinoside (AraC)	Sigma
LPS	Sigma
P3CSK4	Sigma
Glutamine	Sigma
O-(2-Acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate (PUGNAC)	Sigma
Alloxan	Sigma

3.1.2 Viruses

Virus	Supplier
MVA (cloned isolate F6)	Dr. Sutter, G. (LMU)
VACV WR	provide by Dr. Moss, B. (NIH, Bethesda, MD).
VACV Wyeth	provided by Dr. Moss, B. (NIH, Bethesda, MD).

3.1.3 Oligonucleotides

Target Gene	Sequence in 5'-3'orientation	Size (bp)
murine GAPDH-F	GAC AAC TCA CTC AAG ATT GTC AG	540
murine GAPDH-R	GTA GCC GTA TTC ATT GTC ATA CC	
murine TNF-F	CAC TCC CCC AAA AGA TGG	445
murine TNF-R	GAG ATA GCA AAT CGG CTG AC	
murine IFN-β-F	ATG GAA AGA TCA ACC TCA CCT AC	502
murine IFN-β-R	TAG ATT CAC TAC CAG TCC CAG AG	
murine CCL2-F	AAGCCAGCTCTCTCTTCCTC	605
murine CCL2-R	GATTCACAGGAGAGGGAAAAATG	
murine CXCL10-F	TGAAAAAAGAATGATGAGCAGAG	400
murine CXCL10-R	GTACAGAGCTAGGACAGCCATC	
human GAPDH-F	AGCCACATCGCTCAGAACAC	606
human GAPDH-R	GAGGCATTGCTGATGATCTTG	
human IFN-β-F	TGC TCT CCT GTT GTG CTT CTC C	459
human IFN-β-R	CAG TGA CTG TAC TCC TTG GCC TTC	
human CCL2-F	CAA ACT GAA GCT CGC ACT CTC GCC	550
human CCL2-R	GCA AAG ACC CTC AAA ACA TCC CAG	
human CXCL8-F	GTA AAC ATG ACT TCC AAG CTG G	400
human CXCL8-R	AGA CCC ACA CAA TAC ATG AAG TG	
VACV E3L-F	GATCTATATTGACGAGCGTTCTG	201
VACV E3L-R	GTTGTCATAAACCAACGAGGAG	
VACV B15R-F	TGGTATGTCCCAATATTAATGC	545
VACV B15R-R	AAACGTTGTAGCATCTTCTTCC	

3.1.4 Antibodies

rat anti-VACV C7	Hybridoma culture supernatants (Backes, 2010)
mouse anti-GAPDH	Millipore
Secondary horseradish peroxidase (HRP)-conjugated goat anti-rat polyclonal IgG	BioLegend
Secondary HRP-conjugated goat anti-mouse IgG/IgM	Jackson ImmunoResearch

3.1.5 Buffers

DNA loading buffer 5x 0.25% bromophenol blue, 40% (w/v) glycerol in distilled water and 60 µM EDTA
Phosphate buffered saline (PBS) pH 7.4 140mM NaCl , 5.4mM KCl, 9.7mM Na2HPO4x2H2O, 2mM KH2PO4
TAE buffer 50x pH 8.0 2M Tris-acetate, 0.5M NaCl, 50mM EDTA
TBS pH 7.6 150 mM NaCl, l50 mM, Tris-acetate

3.1.6 Cell migration

Reagent/Equipment	Supplier
FACSCanto II	BD Biosciences
MACSQuant VYB	Miltenyi
Microplate Zentrifuge 5810R	Eppendorf
96 well (V bottom for FACS)	Corning Life Sciences

3.1.7 Western Blot and ELISA

Reagent/Equipment	Supplier
Colour plus protein ladder	New England Biolabs
4-20% Criteron TGX stain free gel	Bio-Rad
Laemmli buffer (x4)	Bio-Rad
Mini PROTEAN tetra cell (electrophoresis tank)	Bio-Rad
Trans-Blot Turbo Mini Nitrocellulose transfer pack	Bio-Rad
Trans-Blot Turbo Transfer system	Bio-Rad
Tris Glycine solution (10x)	Bio-Rad
Tween20	Sigma
β-Mercaptoethanol	AppliChem
ChemiDoc MP imager	Bio-Rad
Clarity ECL Susbtrate	Bio-Rad
Image Lab. 5 Software	Bio-Rad
Nonfat dry milk	Applichem
96 well (Maxisorp, flat bottom for ELISA)	Nuc
Sulphuric acid 1 M	Roth
Sunrise microplate reader	Tecan

3.2 Methods

3.2.1 Viruses

VACV strain Western Reserve (WR) and VACV Wyeth were originally provided by Bernard Moss (NIH, Bethesda, MD). The two VACV strains and MVA (cloned isolate F6) were propagated in chicken embryo fibroblasts (CEF). Virus stocks were purified by ultracentrifugation and titrated using standard methodology (Kremer et al, 2012). All the virus stocks were tested for contamination with Mycoplasma by real-time PCR with the kit Venor®GeM-qEP (Minerva Biolabs GmbH, Berlin, Germany).

Viruses were treated with a UV dosage as indicated using the Stratalinker UV Crosslinker 1800 (Stratagene, USA). Viruses were resuspended at $1X10^8$ PFU in cell culture medium were placed on ice and treated with the indicated dosages of UV light.

3.2.2 Cell lines

The Human monocytic cell line (THP-1) and the Baby hamster kidney cells (BHK-21) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany). L929 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml penicillin and streptomycin. To make conditioned medium for the differentiation of bone marrow cells into bone marrow derived macrophages (BMDM), L929 cells were grown for 10 days in medium that was then centrifuged, filtered through a 0.2 µm filter (BD biosciences, San Jose, CA) and stored at -20 °C. The permanent cell lines were systematically tested for mycoplasma contamination.

3.2.3 Isolation of murine bone marrow cells and generation of BM-derived macrophages (BMDM)

Bone marrow cells were flushed from the femurs and tibiae of mice with very-low endotoxin (VLE)-RPMI 1640 medium (Biochrom) and supplemented with 10% FCS. Red cells were lysed using a lysis buffer (R&D Systems) and then the cells were washed and seeded in VLE-RPMI 1640 medium supplemented with 10% FCS, 20% conditioned medium from L929 cells and 100 U/ml penicillin and streptomycin. For removing the fibroblasts, the supernatant containing BM cells was collected after 24 h and the cells re-seeded in VLE-RPMI 1640 medium with supplements. After 4 days, medium was replaced with VLE-RPMI 1640 medium and supplemented with 10% FCS, 10% L929 conditioned medium and standard antibiotics. On day 7 post isolation, macrophages were harvested and seeded to a final density of 0.5×10^6 cells/ml in 6-well and 24-well cell culture plates (SARSTEDT, Nümbrecht, Germany). Differentiation of BMDM was verified by microscopy (Francke et al, 2011).

3.2.4 Analysis of heated-MVA and UV-irradiated MVA growth

BHK21 cells were infected with MVA, heated MVA or UV-irradiated MVA at an MOI of 0.05. After 1 h incubation, cell culture supernatant was replaced with medium containing 2% FCS. At 0 h, 12 h, 24 h and 48 h p.i., cells were harvested by scraping and frozen until analysis. Viral titers were determined by tissue culture infectious dose 50 (TCID50) using Reed-Muench methodology (Reed & Muench, 1937)**.**

3.2.5 Infection of cells and harvest of samples

1.5×10^6 / ml BMDM or 1.0×10^6 / ml THP-1 cells were added to 6 well plates in RPMI 1640 medium supplemented with 1% FCS and infected with MVA or WR at an MOI of 1 and 4 respectively. After 6 h incubation, some cells were lysed for total RNA isolation and protein preparation for Western Blot. The rest of the cells were further

incubated for 20 h, after which supernatants were harvested. Cells were pelleted and supernatants were collected and treated with 1 J of UV for inactivating remaining virus and stored at -80 °C.

3.2.6 RT-PCR

Total RNA was extracted after 6 h incubation, using an RNeasy Plus kit (Qiagen, Hilden, Germany) and then the cDNA was synthetized using Omniscript reverse transcriptase (Qiagen). PCR was carried out as described previously (Lehmann et al, 2002). The oligonucleotides for amplification of human and murine housekeeping genes, specific cytokines as well viral genes were designed using Primer3 software (Rozen & Skaletsky, 2000). Sequences of oligonucleotides and PCR product sizes are included in Materials. The primers were synthesized by Eurofins Genomics (Ebersberg, Germany). PCR products were separated on a 1.5% agarose gel and stained with GelRed™ (Biotium, Hayward, CA). Pictures of gels were taken with ChemiDoc MP System and analyzed using Image Lab software (BioRad).

3.2.7 Western Blot

Cells were lysed in 1 × SDS sample buffer including β-mercaptoethanol (BioRad). Subsequently, cellular lysates were heated at 95°C for 5 min and subjected to sonication for 3 min. Proteins were separated on a 15% Tris-glycine SDS-polyacrylamid gel then subsequently transferred onto a Protran® nitrocellulose membrane (Whatman, Dassel, Germany). Membranes were immunostained using rat anti-VACV C7 mAb diluted 1:200 from hybridoma culture supernatants (Backes, 2010), anti-VACV E3 polyclonal antisera from rabbits 1:1000, and mouse anti-GAPDH mAb (Biolegend) diluted 1:10.000. Secondary horseradish peroxidase (HRP)-conjugated goat anti-rat polyclonal IgG (BioLegend) and HRP-conjugated goat anti-mouse IgG/IgM were diluted 1:20.000 (Jackson ImmunoResearch Laboratories,

Inc., West Grove, PA) and HRP-conjugated mouse anti-rabbit IgG was diluted 1:5000 (Jackson ImmunoResearch Laboratories). Detection reagent solutions were used from Clarify™ Western ECL substrate Kit (Bio-Rad). The detection of O-GlcNAc modified proteins was performed using an O-GlcNAc Western Blot Detection Kit (Thermo Scientific) and carried out following the manufacturer's instructions. Positive signals were detected with the ChemiDoc Imager (Bio-Rad) and analyzed using Image Lab. 5 Software (Bio-Rad).

3.2.8 ELISA

To determine cytokine concentrations in cellular supernatants mouse CCL2 ELISA MAX™ Deluxe (BioLegend), mouse TNF-α ELISA MAX™ Deluxe (BioLegend), human CCL2 ELISA MAX™ Deluxe (BioLegend) and human CXCL8 ELISA MAX™ Deluxe (BioLegend) kits were used and were carried out according to the manufacturer's instructions. Capture antibody was diluted and was added to each well of a 96 well plate (NUNC Maxisorp) sealed and incubated over night at room temperature. The plate was washed 4x with excess PBS containing 0.01% Tween20 and dried on absorbent tissue. The plates were blocked by adding 200 µl of Diluent Assay and incubated at room temperature for 1 h. A serial dilution of standards was prepared and 100 µl each added to the plate, simultaneously with supernatant samples and incubated for 2 h at room temperature. The plate was washed 4x. Next, a biotinylated secondary detection antibody is added. After 1 h incubation at room temperature, the plate was washed 4x and 100µl of Streptavidin-HRP diluted in diluent assay was added to each well, then incubated for 30 min at room temperature. The plate was washed 5x and developed by addition of 100 µl of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) Substrate Solution. The reaction was stopped by addition 100 µl of 1 M sulphuric acid and optical density was measured at 450 nm on a Tecan Sunrise microplate reader. The concentration of the cytokines in

supernatants samples were determined by interpolation from the standard curve using non-linear regression.

3.2.9 Chemotaxis assays

Chemotaxis assays were performed as previously described (Lehmann et al, 2006). THP-1 cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI and allowed to migrate for 30 min in a 96-well Multi-Screen-MIC plate (8 µm pore size, Millipore, Billerica, MA). Cells were incubated with supernatants from THP-1 cells, MVA-infected THP-1 cells or MVA-infected THP-1 cells incubated with Alloxan. The number of transmigrating cells in the bottom chamber was quantified using the MACSQuant VYB flow cytometer (Miltenyi Biotec).

3.2.10 Statistical analysis

All data was assembled using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA), significance was determined by non-parametric Mann-Whitney U test, a *P* value of < 0.05 was considered to be statistically significant.

4. RESULTS

4.1 Presence of early viral RNA correlates with cytokine expression in MVA-infected monocytes and macrophages.

Previously Lehmann *et al.* have shown that MVA but no other VACV strains elicit CCL2 expression in THP-1 cells (Lehmann et al, 2009). However the mechanism of chemokine induction in MVA-infected cells is poorly understood. Therefore physically and chemically treated MVA was investigated to reveal critical steps of the viral replication cycle for induction of cytokine expression.

4.1.1 MVA-induced chemokine expression is independent of viral DNA replication in THP-1 cells.

A number of cytosolic DNA sensors have been reported in the last years, some of them being involved in the recognition of VACV infection (Peters et al, 2013; Yoneyama & Fujita, 2010a). Thus, since poxvirus is a DNA virus the contribution of viral DNA replication for the induction of chemokine expression in MVA infected human monocytic THP-1 cells was tested using cytosine arabinoside (AraC). AraC blocks poxviral DNA replication and consequently transcription of viral intermediate and late genes is inhibited (Taddie & Traktman, 1993). Therefore, as expected, transcription of the late VACV B15R gene but not the early VACV E3L gene was affected by AraC in MVA-infected THP-1 cells (Fig. 1A). Importantly, AraC did not significantly affect CCL2 and CXCL8 mRNA transcription and protein production, indicating that the induction of these chemokines by MVA in THP-1 cells was independent of viral DNA replication (Fig. 1).

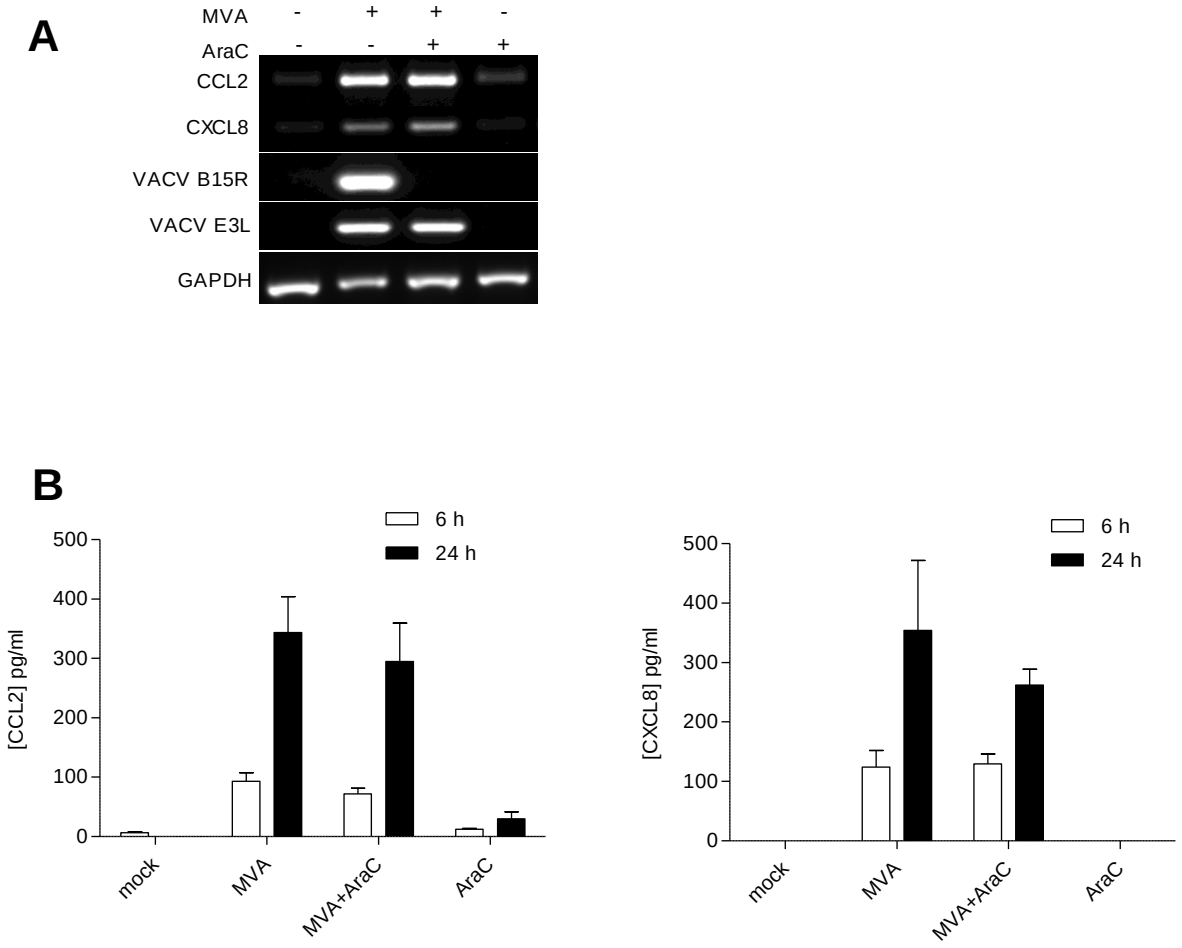

Fig.1. Induction of chemokines in MVA-infected THP-1 cells is independent of viral DNA replication. THP-1 cells were infected with MVA at an MOI of 4. Cells and MVA were pretreated with AraC (50 µg/ml) as indicated. **(A)** Total RNA was isolated 6 h p.i. and analyzed using specific RT-PCR. **(B)** CCL2 and CXCL8 protein concentrations in the cell culture supernatants were determined by ELISA at 6 h p.i and 24 h p.i. The data shown are representative of two independent experiments. Errors bars indicate standard deviations (SD).

4.1.2 Inhibition of early viral protein synthesis does not affect cytokine production in MVA and VACV-infected cells.

Since AraC has no effect on the synthesis of early viral mRNA and protein (Taddie & Traktman, 1993), the relevance of early viral protein expression for cytokine induction in MVA-infected cells was investigated using adenosine N1-oxide (ANO). ANO blocks translation of VACV early mRNAs, without affecting cellular protein synthesis (Kane & Shuman, 1995).

Firstly, the effect of ANO on the synthesis of VACV C7, an early viral protein, was tested. Prior to infection THP-1 cells and MVA were pre-incubated with ANO for four and one hour, respectively. This led to a reduction of VACV C7 protein levels by 70% as compared to non-treated MVA-infected THP-1 cells. The residual VACV-C7 protein detected by WB is probably caused by the viral inoculum. (Fig. 2A,B). As expected, ANO blocked the transcription of the late viral gene VACV B15R but not the transcription of the early viral gene VACV E3L or the housekeeping gene GAPDH (Fig. 2C,D). As determined by RT-PCR, ANO increased the levels of cytokine mRNAs of IFN-β and CCL2 in MVA-infected THP-1 cells (Fig. 2C,D). CCL2 and CXCL8 protein production was strongly upregulated in the presence of ANO in MVA-infected THP-1 cells (Fig. 2E). Interestingly, the secretion of CCL2 was induced in VACV WR-infected THP-1 cells in the presence of ANO (Fig. 2E). These results indicates that early viral protein synthesis rather inhibits than induces cytokine expression in THP-1 cells.

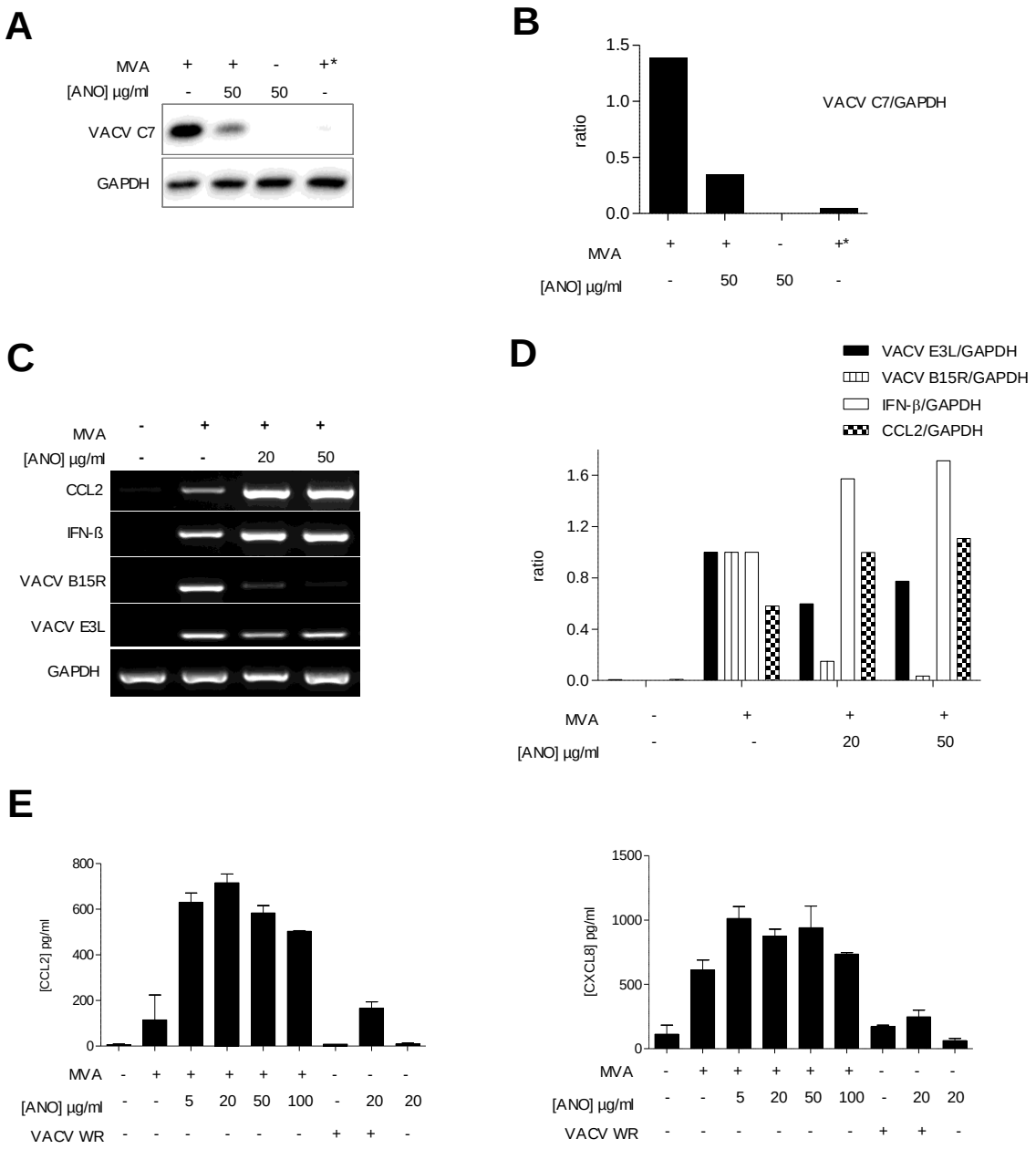

Fig.2. MVA-induced cytokines in THP-1 infected cells is independent of viral early protein translation. THP-1 cells were infected with MVA or VACV WR at an MOI of 4. Where indicated cells were pretreated with ANO at concentrations as indicated. **(A)** Cells were lysed 6 h p.i. and protein was analyzed with a VACV C7L specific Western Blot. GAPDH was used to demonstrate equal protein loading. Where indicated (*), protein extraction was performed at 0 h p.i. **(B)** Ratio of the VACV C7L protein band intensity to GAPDH. **(C)** Total RNA was isolated 6 h p.i. and analyzed using specific RT-PCRs as indicated. **(D)** Ratio of viral gene and cytokine band intensities to GAPDH. **(E)** Protein concentrations of CCL2 and CXCL8 was determined in the cellular supernatants by ELISA 24 h. p.i. The data shown are representative of two independent experiments. Errors bars indicate SD.

4.1.3 MVA and VACV-Wyeth treated with limited dosages (0.2-0.8 J) of UV induce cytokine expression in the human monocytic cell line THP-1 and BMDM.

The previous results indicate that the critical signal for induction of cytokine expression triggered by the virus is before viral protein translation starts.

Therefore, the importance of viral nucleic acids for triggering cytokine production was investigated in THP-1 cells and BMDM. MVA and VACV were irradiated with different dosages of UV light, a method commonly used for blocking viral nucleic acid synthesis. Firstly the dosage of UV light used to irradiate MVA and its affects on replication were determined. As shown in figure 3, MVA irradiated with UV-light at 0.8 and 3.2 J was unable to productively replicate in BHK21 cells, which is permissive for MVA replication (Fig. 3).

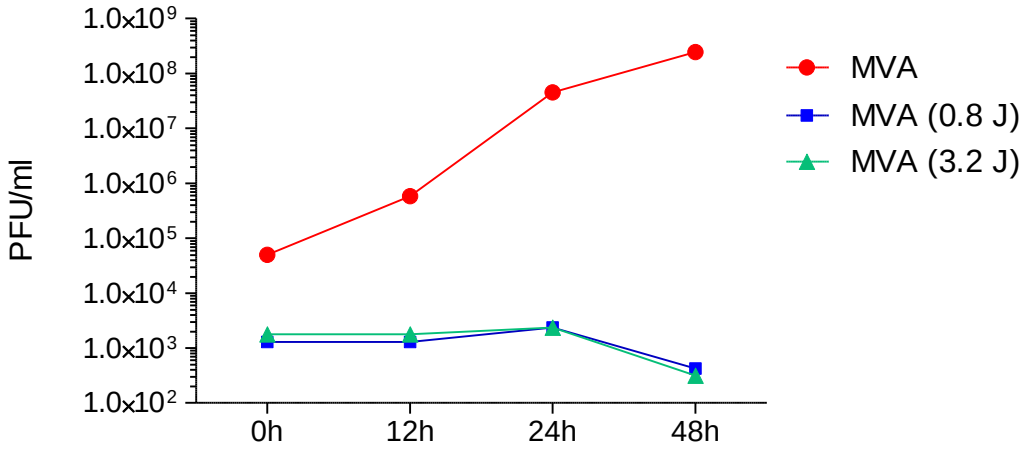

Fig.3. UV-treated MVA does not replicate in BHK21 cells. (A) To measure virus replication, BHK21 cells were infected with MVA or UV-treated MVA at an MOI of 0.05. Virus titers were determined by TCID50 at the indicated time points.

Furthermore, even a dosage of 0.2 J was sufficient to nearly completely prevent early viral protein synthesis in THP-1 cells (Fig. 4C,D) and BMDM (Fig. 5C,D). In contrast, a dosage higher than 0.8 J was necessary to prevent detection of early viral RNA by RT-PCR in THP-1 cells (Fig. 4A,B), however in BMDM (Fig. 5A,B), even a dosage of 1.6 J was not sufficient.

When the dosage of UV was sufficient (1.6 J) to reduce viral mRNA to a level where it was no longer detectable by RT-PCR, CCL2, CXCL8 and IFN-β mRNA were reduced to the levels of mock-infected THP-1 cells (Fig. 4A,B). Of note, treatment of MVA with UV light at 0.2 J and 0.4 J led to enhanced induction of chemokines in THP-1 cells (Fig. 4A,B,E).

In BMDM, treatment of MVA with UV light at a dosage of 0.2 J was sufficient to prevent detection of the late VACV B15R mRNA by RT-PCR. In contrast, even if MVA was treated with UV light at a dosage of 1.6 J the early VACV E3L mRNA as well as cytokines were still detected.

CCL2 and TNF-α protein concentrations were increased in supernatants of BMDM infected with MVA treated with UV light at a dosage of 0.2 J and 0.4 J as compared to supernatants of BMDM infected with non-treated MVA (Fig. 5E).

The wild type strains VACV Wyeth and VACV WR did not trigger cytokine expression in THP-1 cells and BMDM, respectively. However, interestingly, when VACV was treated with UV light at low dosages (0.2-0.8) then it gained the ability to elicit cytokine production in THP-1 cells (Fig. 4E) and BMDM (Fig. 5E).

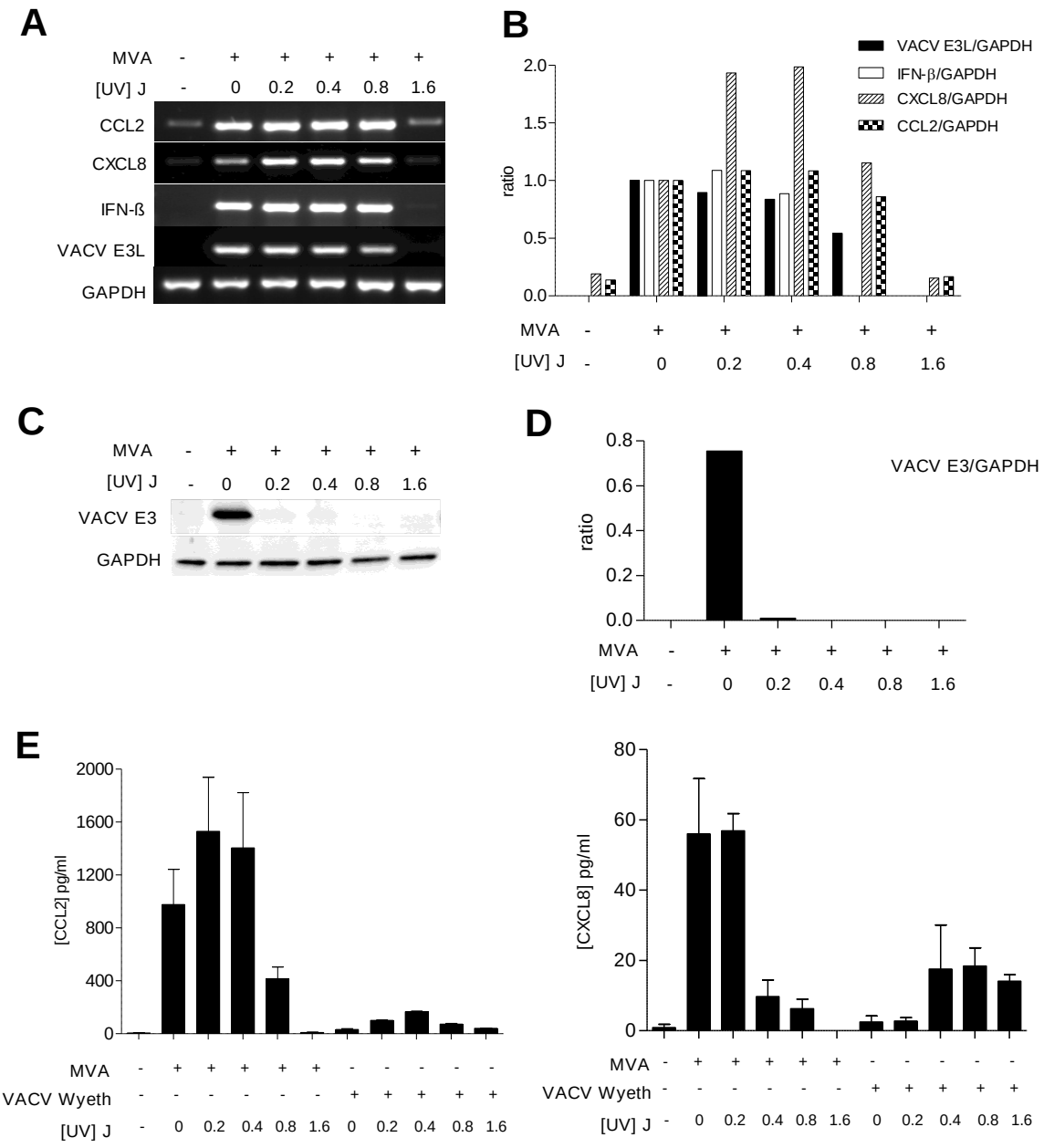

Fig.4. Effect of UV light on MVA and VACV Wyeth induced cytokine expression in THP-1 cells. THP-1 cells were infected with MVA or VACV Wyeth at an MOI of 4. Viruses were irradiated with UV light at dosages as indicated. **(A)** Total RNA was isolated 6 h p.i. and analyzed using specific RT-PCR. **(B)** Ratios of VACV E3L and cytokine band intensities to GAPDH band intensities. **(C)** Cells were lysed 6 h p.i. and analyzed with a VACV E3L specific Western Blot. GAPDH was used to demonstrate equal protein loading. **(D)** Ratios of VACV E3L protein band intensities to GAPDH protein band inensities. **(E)** CCL2 and CXCL8 protein concentrations were determined by ELISA in the cellular supernatants collected 24 h p.i. The data shown are representative of two independent experiments. Errors bars indicate SD.

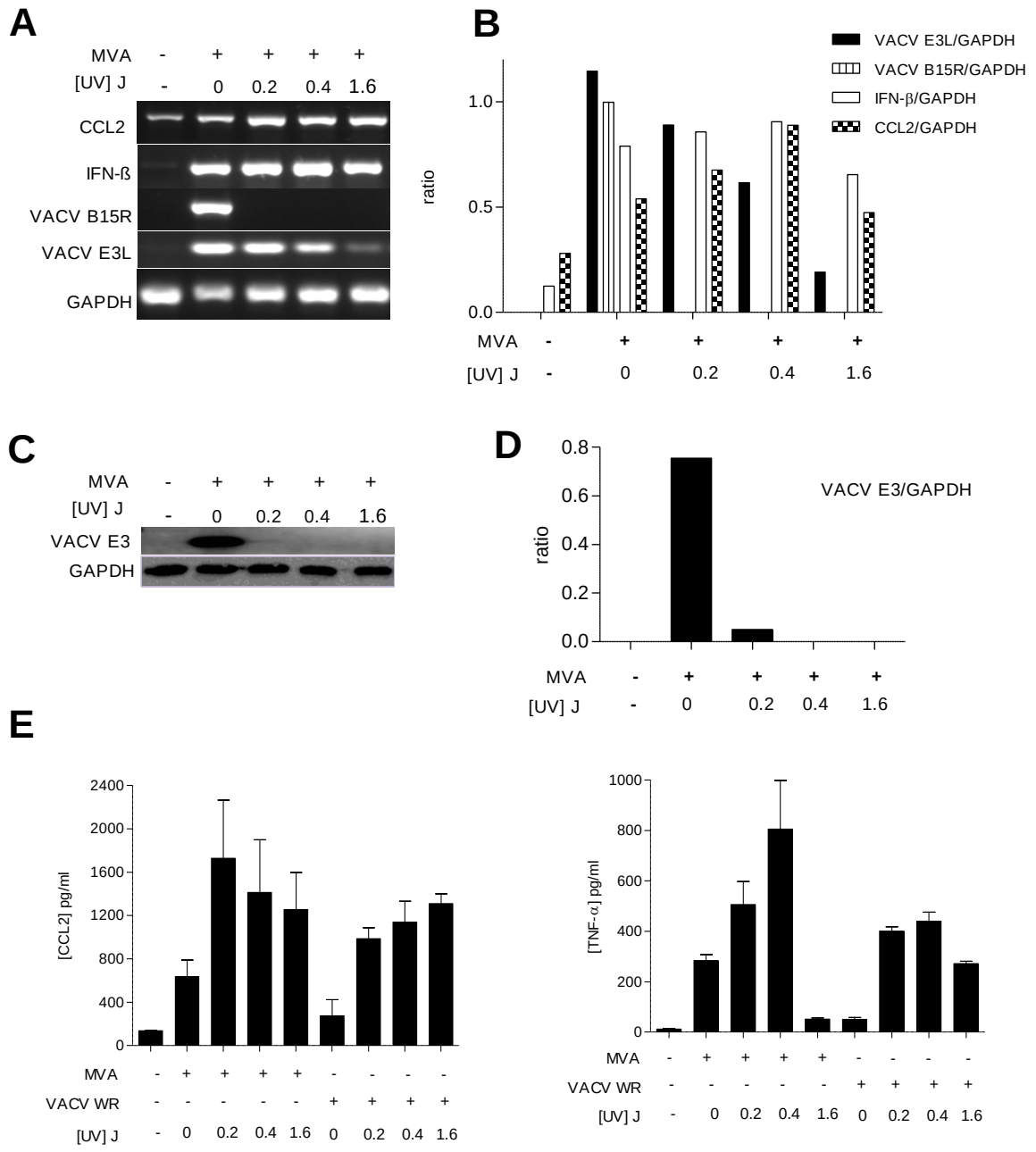

Fig.5. MVA and VACV WR treated with UV light induce cytokine expression in BMDM. BMDM were infected) with MVA and VACV WR, at an MOI of 1. Viruses were irradiated with UV light at dosages as indicated. **(A)** Total RNA was isolated 6 h p.i. and analyzed using specific RT-PCR. **(B)** Ratios of VACV E3L and cytokine band intensities to GAPDH band intensities. **(C)** Cells were lysed 6 h p.i. and analyzed by a VACV E3L specific WB. GAPDH was used to demonstrate equal protein loading. **(D)** Ratios of VACV E3L protein band intensities to GAPDH protein band intensities. **(E)** CCL2 and TNF-α protein concentration were determined by ELISA in the cellular supernatants collected 24 h p.i. The data shown are representative of two independent experiments. Errors bars indicate SD.

4.1.4 Toll-like receptor 2 and 4 do not mediate MVA induced CCL2 and IFN-β expression in BMDM.

Previously, a role for TLR2 in sensing VACV has been proposed (Barbalat et al, 2009; O'Gorman et al, 2010; Zhao et al, 2009; Zhu et al, 2007). Therefore, the capability of MVA to induce cytokine expression in TLR2/TLR4-deficient ($TLR2^{-/-}$/$TLR4^{-/-}$) BMDM was investigated.

First, wild type BMDM were infected with MVA or stimulated with P_3CSK_4 or LPS which are ligands for TLR2 and TLR4, respectively. RT-PCR revealed that MVA, P_3CSK_4 and LPS increased CCL2 mRNA to nearly equal levels in wild type BMDM. IFN-β mRNA was only induced by MVA (Fig. 6A,B). As expected, P_3CSK_4 and LPS did not increase CCL2 mRNA levels in $TLR2^{-/-}$/$TLR4^{-/-}$ BMDM. Importantly, MVA increased mRNA levels of CCL2 and IFN-β as compared to the levels detected in cells challenged with P_3CSK_4 and LPS (Fig. 6C,D). As shown in wild type BMDM (Fig. 5), VACV treated with UV light gained the capability to elicit cytokine production in $TLR2^{-/-}$/$TLR4^{-/-}$ BMDM (Fig. 6C,D). Additionally, treatment of MVA and VACV with UV light until a dosage of 0.8 J did not prevent early viral mRNA and cytokine mRNA detection by RT-PCR (Fig. 6 C,D). Thus, TLR2 and TLR4 play no role in MVA induced cytokine expression in BMDM.

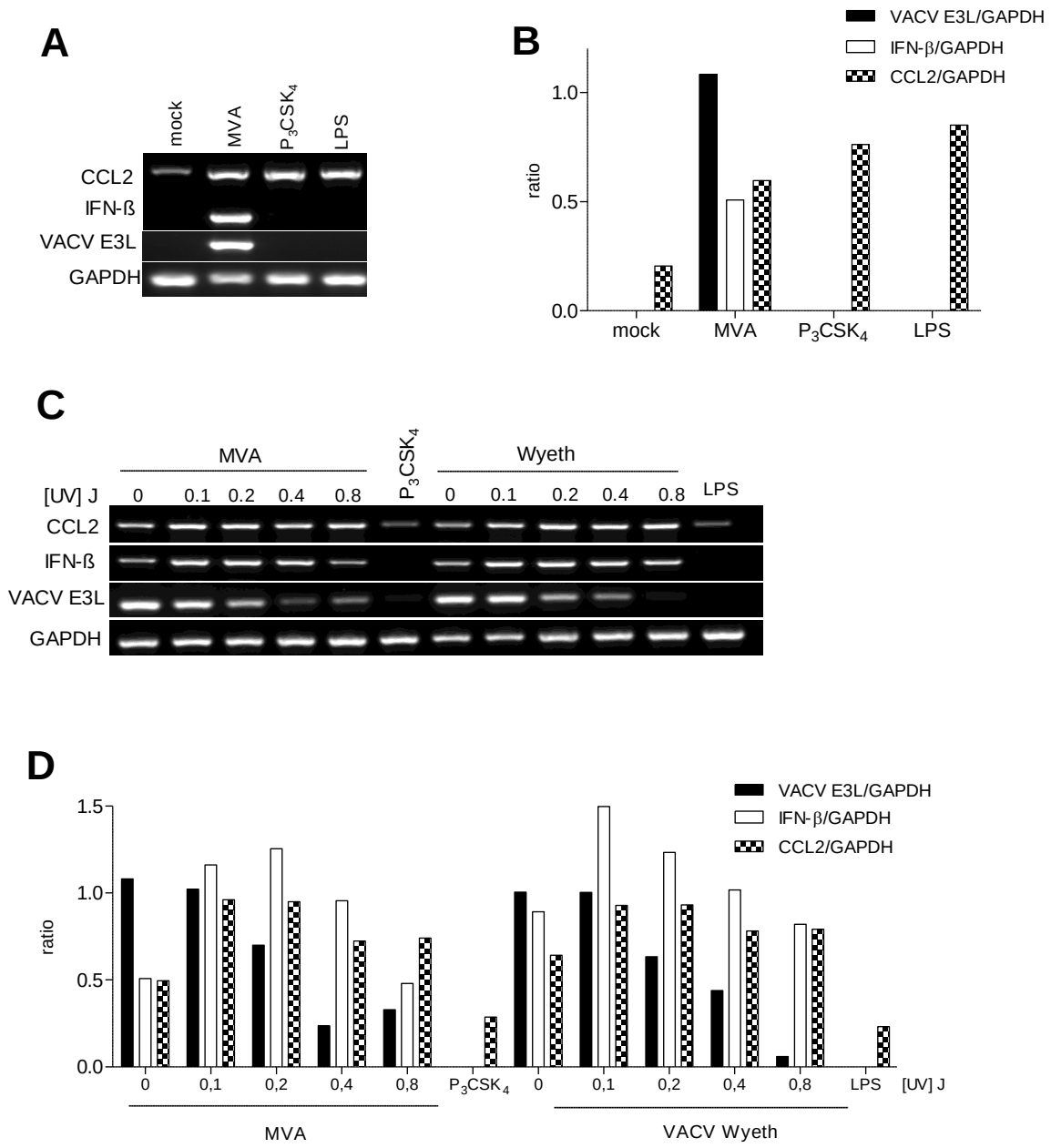

Fig.6. Effect of UV light on MVA and VACV Wyeth increased CCL2 and IFN-β **mRNA levels in TLR2$^{-/-}$/TLR4$^{-/-}$ BMDM.** BMDM from wild type and TLR2$^{-/-}$/TLR4$^{-/-}$ mice were infected with MVA or VACV Wyeth at an MOI of 1 or challenged with P_3CSK_4 (1 µg/ml) or LPS (1 µg/ml). Viruses were irradiated with UV light at dosages as indicated. **(A)** Total RNA from wild type BMDM and **(C)** TLR2$^{-/-}$/TLR4$^{-/-}$ BMDM were isolated 6 h p.i. and analyzed using specific RT-PCR. **(B, D)** Ratios of VACV E3L and cytokine band intensities to GAPDH band intensities.

4.1.5 MVA heated at 55°C does not affect cytokine expression in MVA-infected THP-1 cells and BMDM.

The previous results indicate that the presence of early viral mRNA correlates with the capability of MVA and VACV, which was treated with UV light, to induce cytokine expression. Since it cannot be excluded that treatment with high dosages of UV light has additional affects beyond the inhibition of viral mRNA transcription, viruses were also treated with heat which prevents detection of viral mRNA by RT-PCR in infected cells while preserving the integrity of some functional proteins in the viral particle (Harper et al, 1978). Heat-treatment of VACV at 55°C for 1 h selectively affects the activity of the viral capping enzyme which is present in the virion and required for termination of viral transcription (Broyles, 2003).

Treatment of MVA at 55°C for 1 h or at 75°C for 20 min abrogated its replication in the permissive cell line BHK21 (Fig. 7) and completely blocked early viral protein synthesis in THP-1 cells (Fig. 8B) and BMDM (Fig. 9B).

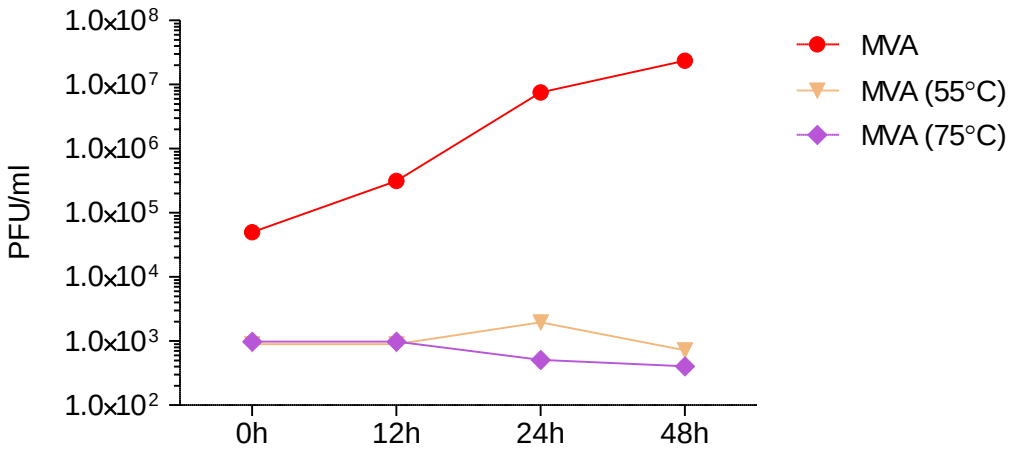

Fig.7. Heat treated MVA does not replicate in BHK21 cells. BHK21 cells were infected with MVA at an MOI of 0.05. Where indicated, MVA was treated at 55°C or 75°C for 1 h or 20 min, respectively. Virus titers were determined by TCID50 at the indicated time points.

As expected, heating of MVA at 75°C for 20 min completely blocked its capability to trigger cytokine mRNA transcription (Fig. 8A and Fig. 9A). However, heat treatment of MVA at 55°C for 1 h did not affect its capability to induce cytokine mRNA synthesis and protein production in THP-1 cells (Fig. 8A,C,D) and BMDM (Fig. 9A,C,D). Furthermore, it was confirmed that VACV WR does not induce cytokine production in THP-1 cells (Fig. 8C,D) and BMDM (Fig. 9C,D). However, CCL2 expression was strongly enhanced in BMDM infected either with MVA or VACV WR heated at 55°C for 1 h (Fig. 9C,D).

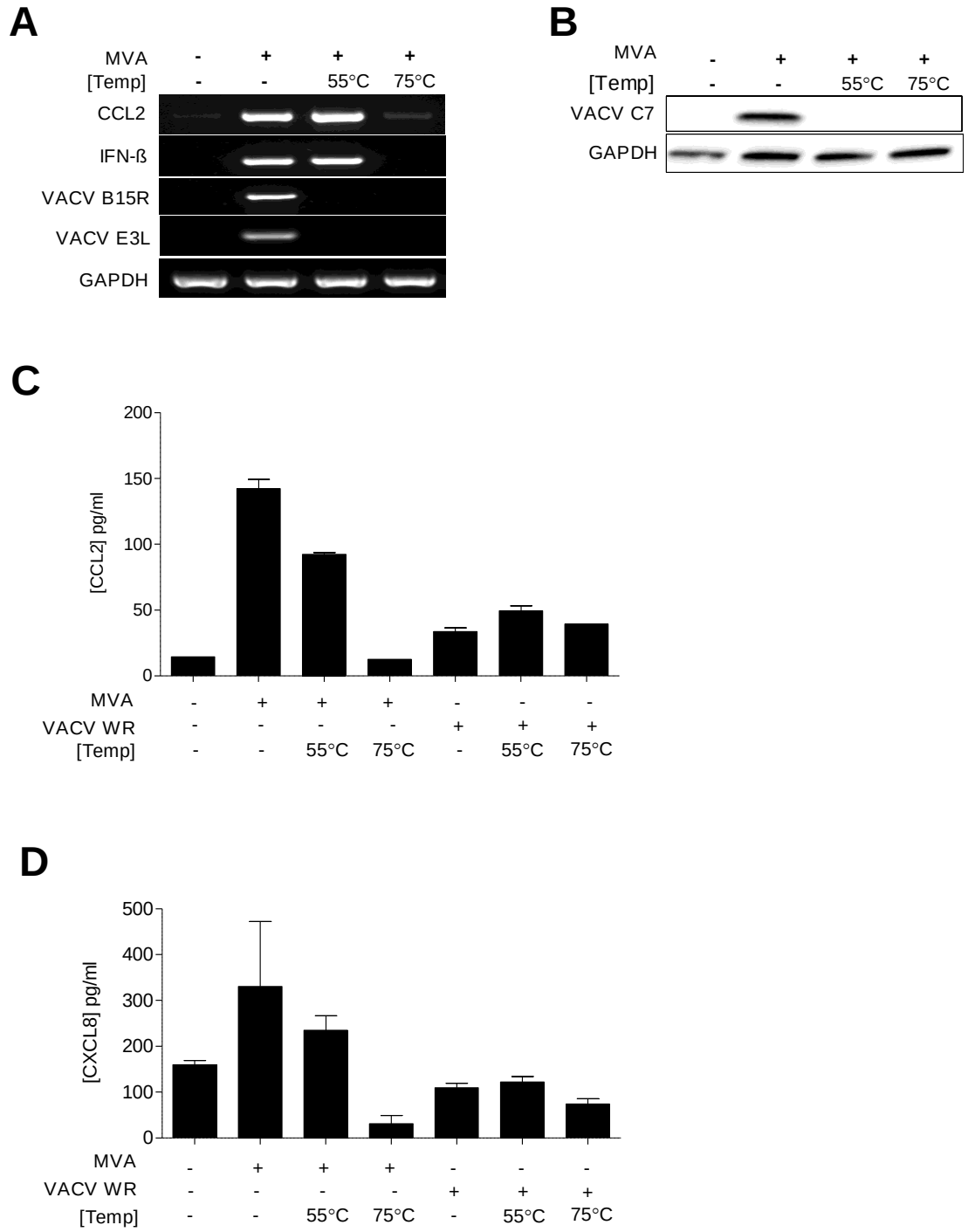

Fig.8. MVA heated at 55°C for 1 h induces cytokine expression in THP-1 cells. THP-1 cells were infected with MVA at an MOI of 4. **(A)** Total RNA was isolated 6 h p.i. and analyzed using specific RT-PCR as indicated. **(B)** Cells were lysed 6 h p.i. and analyzed with a VACV C7L specific Western Blot. GAPDH was used to demonstrate equal protein loading. **(C)** CCL2 and **(D)** CXCL8 protein concentration in the cellular supernatants collected 24 h p.i. were determined by ELISA. The data shown are representative of two independent experiments. Errors bars indicate SD.

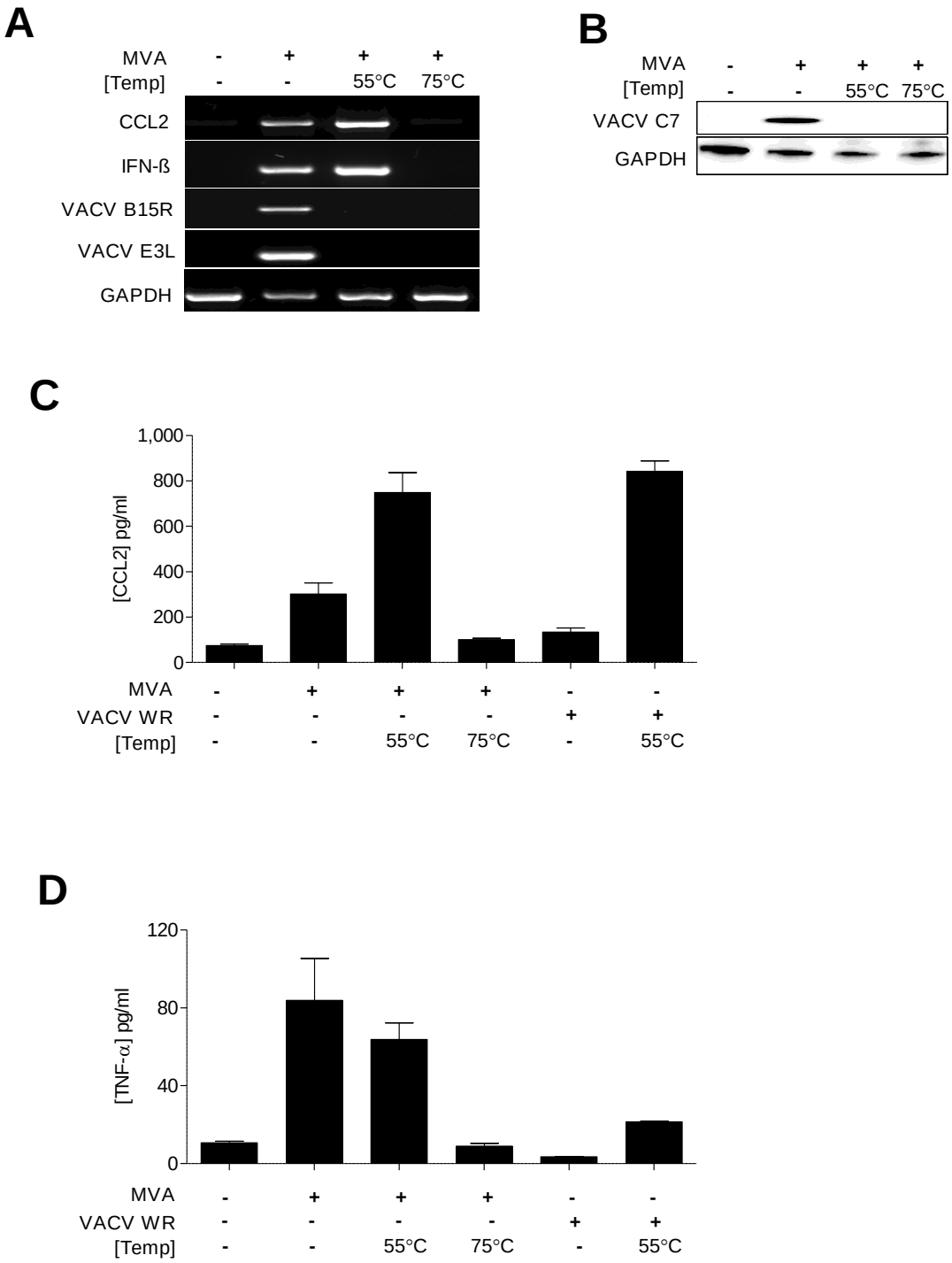

Fig.9. MVA heated at 55°C for 1 h induces cytokine expression in BMDM. BMDM were infected with MVA at an MOI of 1 **(A)** Total RNA was isolated 6 h p.i. and analyzed using specific RT-PCR. **(B)** Cells were lysed 6 h p.i. and analyzed with a VACV C7L specific Western Blot. GAPDH was used to demonstrate equal protein loading. **(C)** CCL2 and **(D)** TNF-α protein concentration in the cellular supernatants collected 24 h p.i. were determined by ELISA. The data shown are representative of two independent experiments. Errors bars indicate SD.

4.2 Type I interferon receptor (IFNAR) is involved in CCL2 expression of MVA-infected BMDM.

4.2.1 Kinetic analysis of cytokine expression in MVA-infected BMDM.

To study the temporal sequence of viral and cytokine mRNA induction in MVA infected BMDM, a kinetic analysis was performed. The expression of the housekeeping gene GAPDH was used as control and the levels of each RNA investigated were determined always also in mock-infected cells.

Early viral gene expression as indicated by VACV E3L transcripts was detected at 1 h p.i. while late gene transcription as indicated by VACV B15R RNA was detected at 3 h p.i., which further increased until 8 h p.i. (Fig. 10 A,C). This represents the typical temporal pattern of VACV transcription. IFN-β expression was detected already at 2 h p.i, preceding chemokine expression (Fig. 10 A,C). Up-regulation of CCL2 and CXCL10 mRNA by MVA was detected as early as 4 h p.i. (Fig. 10 A,C).

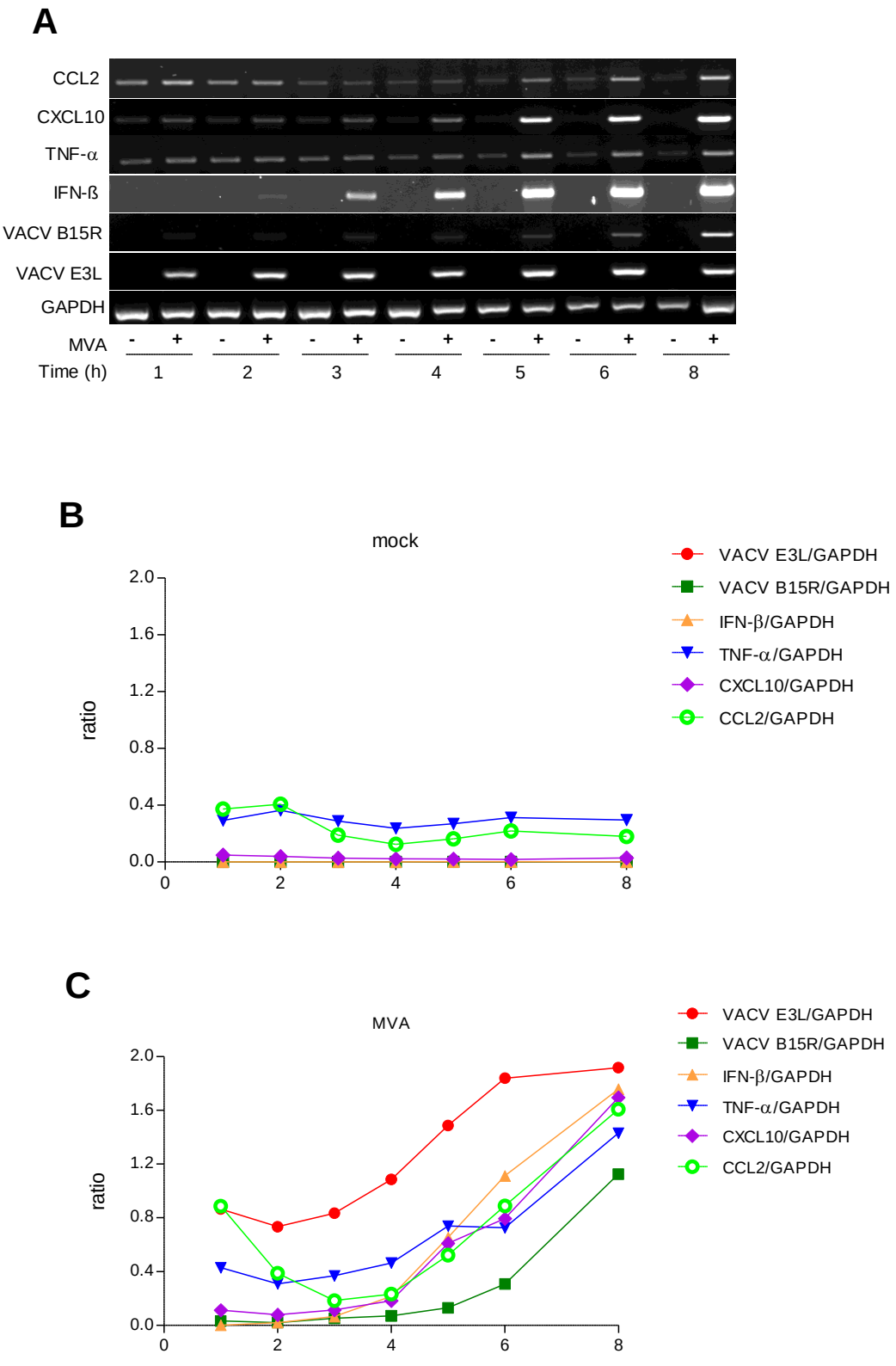

Fig.10. MVA-infection profile in macrophages. **(A)** BMDM were infected with MVA at an MOI of 1, total RNA was isolated after infection at times as indicated and analyzed using specific RT-PCR. **(B)** Ratio of viral and **(C)** cytokine PCR product intensities towards GAPDH PCR product intensity.

4.2.2 MVA-induced CCL2 expression in BMDM is modulated by IFNAR

TNF-α and type I IFNs induce CCL2 expression (Conrady et al, 2013; Sung et al, 2002), yet the kinetic analysis of cytokine expression in MVA-infected BMDM indicated that the activation of IFN-β mRNA transcription but not of TNF-α mRNA precedes the induction of CCL2. Taken together, type I IFNs constitute potential candidates that may induce CCL2 in MVA-infected BMDM. Therefore, the role of the type I interferon receptor (IFNAR) for CCL2 expression was tested using BMDM from mice deficient for this receptor (IFNAR-/-). TNF-α and IFN-β mRNA but no CCL2 mRNA were induced in MVA-infected BMDM from IFNAR-/- mice, and the level was dependent on the MOI applied (Fig. 11). The lack of CCL2 expression was confirmed at the protein level by ELISA in MVA infected BMDM from IFNAR-/- mice (Fig. 11E). This indicates that IFNAR is involved in MVA induced CCL2 expression in BMDM.

In contrast, the level of TNF-α expression was similar in MVA-infected BMDM of wild type and IFNAR-/- mice (Fig. 11F). The TLR2 agonist P_3CSK_4 induced CCL2 as well as TNF-α transcription and protein production in both MVA-infected BMDM of wild type and IFNAR-/- mice (Fig. 11E,F).

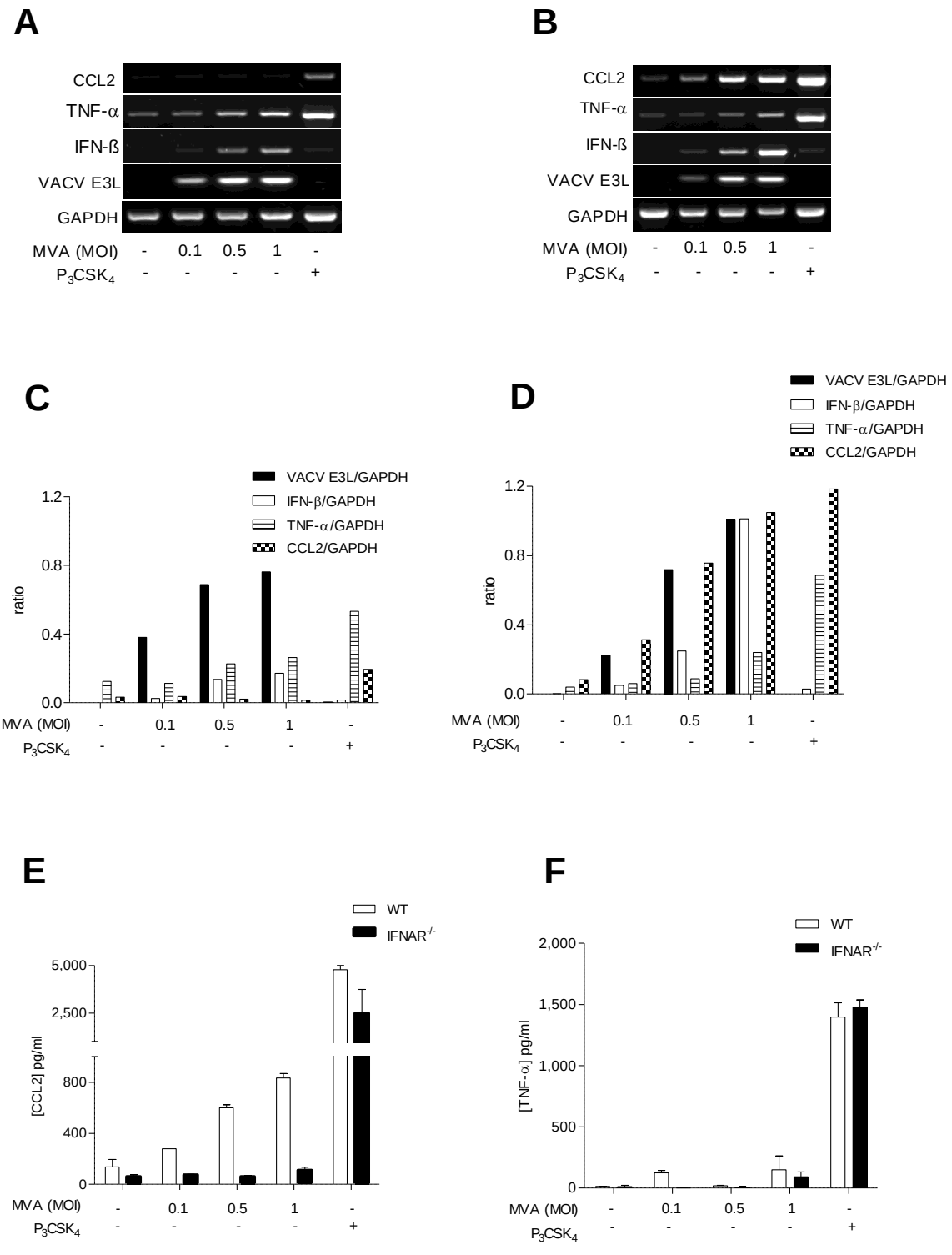

Fig.11. MVA-induced CCL2 expression depends on IFNAR. (A) BMDM from C57BL/6 (WT) and **(B)** IFNAR-/- mice were infected with MVA at an MOI of 1. Total RNA was isolated at 6 h p.i. and analyzed using specific RT-PCR. **(C)** CCL2 and **(D)** TNF-α concentration in the cellular supernatants of BMDM collected 24 h p.i. were determined by ELISA. The data shown are representative of three independent experiments. Errors bars indicate SD.

4.3 O-GlcNacylation is involved in the induction of CCL2 expression in MVA-infected cells.

The promoters of many inflammatory cytokines are controlled by the transcription factor NF-κB (Sung et al, 2002), and NF-κB is activated by MVA (Martin & Shisler, 2009). The activity of NF-κB is modulated by reversible attachment of N-acetyl-glucosamine (O-GlcNAc) on the hydroxyl group of serine and threonine (Golks et al, 2007). The O-GlcNAcylation pattern of intracellular proteins in MVA-infected THP-1 cells was different to non-infected cells (Fig. 12A). Therefore, the requirement of O-GlcNAcylation for induction of CCL2 production in MVA-infected THP-1 cells was investigated. The analysis was done in presence of the O-GlcNAcase (OGA) inhibitors glutamine and O-(2-Acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate (PUGNAC), which prevent removal of O-GlcNAc on the proteins. The GlcNAc residue is attached to proteins by O-linked N-acetylglucosamine transferase (OGT) (Hart et al, 2011), and alloxan is an inhibitor of OGT (Lee et al, 2006). Therefore, modulation of CCL2 expression in MVA-infected cells by alloxan treatment was investigated. Indeed, alloxan decreased CCL2 production in MVA-infected THP-1 cells in a dose dependent manner (Fig. 12B).

As Lehmann *et al.* have shown that the MVA-induced CCL2 expression in THP-1 cells promotes the migration of monocytes and macrophages (Lehmann et al, 2009) the relevance of O-GlcNAcylation in MVA-infected THP-1 cells for inducing cell migration of naïve THP-1 cells was investigated. As shown in figure 12C, migration of naïve THP-1 cells towards supernatants of MVA-infected THP-1 cells treated with alloxan was decreased as compared to supernatants from non-treated, naïve THP-1 cells.

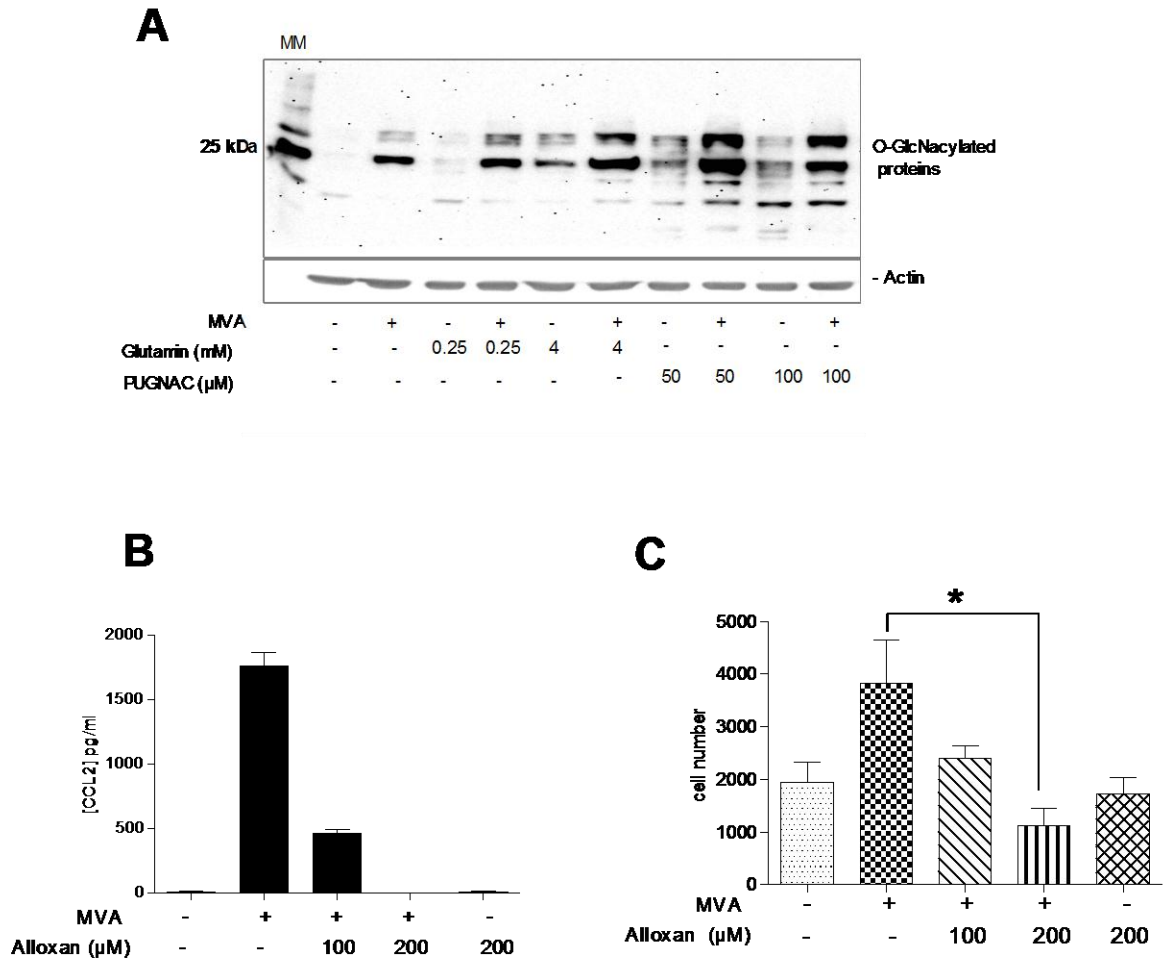

Fig.12. O-GlcNAcylation is required for MVA-induced chemokine expression in THP-1 cells. **(A)** THP-1 cells were infected with MVA and incubated in the presence or absence of glutamine and PUGNAC as indicated. Cells were lysed 8 h p.i. and analysed Western Blot.. Actin was used to demonstrate equal protein loading. **(B)** CCL2 concentration in the cellular supernatants of THP-1 cells collected 24 h p.i. were determined by ELISA. Errors bars indicate SD **(C**) Number of THP-1 cells that migrated towards cell culture supernatants of mock or MVA-infected THP-1 cells in the presence or absence of alloxan. Data are means ± SEM, (n ≥ 3). *,P< 0.05.

5. DISCUSSION

In the last years several recombinant VACV have been designed as viral vectors to produce vaccines against a broad number of infectious diseases (Gilbert, 2013). As a consequence, interest in understanding the interaction of VACV with the immune system has been renewed. The highly attenuated MVA is one of the most promising poxviral vectors, particularly because it is able to induce rapid local immune responses that might enhance its efficacy as a vaccine (Cottingham & Carroll, 2013; Gilbert, 2013; Price et al, 2013). Although the improved ability of MVA versus VACV in triggering immunogenic signaling is well known, the mechanisms of sensing MVA are not totally characterized. Therefore this study attempted to better understand how MVA triggers intracellular signaling.

Since VACV is a DNA virus, the contribution of viral DNA replication for inducing cytokine expression in a monocytic cell line was evaluated. Inhibition of viral DNA replication did not decrease MVA-induced cytokine production in THP-1 cells. This result is in line with that obtained by Waibler *et al*., who reported that blocking viral DNA replication with AraC did not decrease the secretion of IFN-α in MVA-infected bone marrow plasmacytoid dendritic cells (BM-pDC) (Waibler et al, 2007). In contrast, it was reported that exposure of MVA-ΔE3L-infected keratinocytes to AraC at concentrations sufficient to block virus replication also abrogated IFN-β, IL-6, CCL4 and CCL5 production (Deng et al, 2008). Thus, it must be considered that the sensing of the virus is different in these cell types. Recently, several DNA sensors for VACV infection have been described (Yoneyama & Fujita, 2010b). Specifically, the adaptor molecule stimulator of interferon genes (STING) and the newly discovered DNA sensor cyclic GMP-AMP synthase (cGAS) seem to play a key role in the

recognition of viral DNA in VACV-infected cells (Ablasser et al, 2013). Furthermore, Dai *et al.* found that MVA induction of type I IFN was totally dependent on STING and cGAS, and in agreement with our results the induction of IFN in BMDC was independent of viral DNA replication (Dai et al, 2014). Another mechanism of sensing cytosolic viral DNA is mediated by RNA polymerase III, enzyme that synthesizes 5´ppp RNA and induces type I IFN through the RIG-I and MAVS pathway (Chiu et al, 2009).

Previously it was proposed that early VACV proteins activate NF-κB, a key transcription factor necessary for expression of many inflammatory cytokines (Martin & Shisler, 2009). Therefore, early viral protein production was blocked by addition of ANO in VACV-infected cells and the effect on cytokine expression was investigated. The drug ANO substitutes ATP during RNA synthesis by viral RNA polymerase and blocks translation of viral proteins, without affecting cellular protein synthesis (Kane & Shuman, 1995). In the present study, synthesis of the early viral protein C7 was reduced more than 70% in the presence of ANO as compared with non-treated MVA-infected cells. Previously, it was reported that ANO completely prevents early VACV protein production as detected by [^{35}S]methionine pulse-labeling, which can only detect *de novo* synthesized polypeptides (Kane & Shuman, 1995). In this study detection of viral protein was done by WB enabling detection of viral proteins which are also present in the virus preparation, as demonstrated by VACV C7.

Importantly, cytokine secretion was enhanced in MVA and VACV WR-infected THP-1 cells which were treated with ANO. This finding also confirms the previous observation that MVA-induced cytokine expression was independent of DNA replication, since early viral protein synthesis is required for the release of the viral genome prior to DNA replication (Mercer et al, 2012). In contrast, Martin and Shisler

claimed that early viral protein synthesis is necessary for NF-κB activation in MVA-infected 293T fibroblast cells (Martin & Shisler, 2009). However, in that study cordycepin was used to block early viral protein synthesis, and cordycepin has been reported to exert many other effects such as (i) blocking of NF-κB activation (Lee et al, 2009), (ii) inhibition of reactive oxygen species production (Won et al, 2009), (iii) increase of cyclic AMP and cyclic GMP concentration (Cho-HJ 2007 Eur. J Pharmacol) and (iv) induction of the anti-inflammatory cytokine IL-10 (Zhou et al, 2002).

Also UV light can be used to inactivate VACV, most probably by blocking viral nucleic acid production, though formation of thymidine dimers, with minimal effects on viral surface proteins (Tsung et al, 1996). In order to explore the contribution of viral nucleic acids to cytokine induction, VACV was irradiated with UV light at different dosages. The current study shows that MVA treated with high dosages of UV light (4 J) does not induce cytokine production in MVA-infected THP-1 cells and MVA-infected BMDM. These results are similar to a previous study, which showed that treatment of MVA with UV light for 20 min prevented CCL2 secretion *in vitro* and *in vivo* (Lehmann et al, 2009). On the other hand, chemokine production was enhanced in cells infected with MVA or VACV treated with low dosages of UV light (0.25-0.8 J), which is in line with the findings that MVA irradiated with low dosages of UV light enhanced IFN-α response in BM-pDC and BM-mDC (Waibler et al, 2007). Additionally, Drillien et al. observed that irradiated MVA is still able to activate human DC, measured by the up-regulation of the activation marker CD86 (Drillien et al, 2004). The current study demonstrates that enhanced cytokine production correlates with the synthesis of early viral mRNA. Previously, it has been shown that UV treatment of VACV for two minutes results in inactivation of viral replication without affecting early viral transcription (Tsung et al, 1996), which is in agreement with the

present findings that low dosages of UV (0.25-0.8 J) do not affect early viral transcription, but prevent early viral protein translation and consequently viral replication. Therefore, a few crosslinks in the large genome of VACV might inhibit viral replication without affecting viral early mRNA transcription that occurs inside the core.

VACV encodes a myriad of proteins involved in host evasion, and many of them inhibit signaling pathways that lead to the production of IFNs and cytokines in the infected cells (Seet et al, 2003). Previously it has been reported that the capability of VACV to block IFN-α production is conferred mainly by early viral proteins (Waibler et al, 2009). Thus, inhibition of early viral protein expression, while preserving early mRNA transcription might be one reason for enhanced cytokine production by cells infected with VACV treated with low dosages of UV, since the virus may have lost immunomodulatory proteins able to block the innate immune response. Another fact to be considered is that VACV irradiated with high dosages of UV light is still able to enter the cell (Tsung et al, 1996). Thus, only the fusion of the VACV virion with the cell surface and entrance to the cytosol seem to be not sufficient to trigger cytokine expression, in contrast with the report about herpes simplex virus (HSV)-1, in which virus-cell fusion was sufficient for triggering innate immunity in a STING-dependent manner (Holm et al, 2012). On the other hand, it would be interesting to test whether MVA or VACV irradiated with low dosages of UV are still able to protect mice against a lethal challenge with mousepox or VACV WR.

The present study raised concerns that the use of UV-inactivated VACV could lead to false interpretations, regarding TLR2-mediated cytokine secretion, when the experiments are not suitably controlled. Particularly, UV-treatment of VACV has been used to claim the relevance of TLR2 for sensing VACV infection (Barbalat et al,

2009). However, most of these studies have not checked for possible residual gene transcription, which could potentially be involved in triggering cytokine expression.

In order to test the relevance of TLR2 in mediating the enhanced cytokine production induced by irradiated VACV, BMDM from TLR2$^{-/-}$/TLR4$^{-/-}$ mice were infected. There was no difference in cytokine production from wild type BMDM and TLR2$^{-/-}$/TLR4$^{-/-}$ BMDM infected with MVA irradiated with UV. This finding indicates that the enhanced cytokine production was independent of the presence of TLR2 and TLR4. While some studies have claimed the importance of TLR2 for sensing VACV, other groups could not confirm this hypothesis (Davies et al, 2014; Zhao et al, 2009). TLR2 has been typically associated with bacteria ligands, and reports showing their relevance as viral sensors in VACV infection are highly contradictory. Thus, as it was mentioned above, there is concern that some of those results are due to contaminations that could potentially lead to false interpretations (Davies et al, 2014; Price et al, 2013).

To further investigate which viral components are important for inducing cytokine expression in MVA-infected cells, MVA and VACV were heated at 55°C for one hour or at 75°C for 20 min prior to infection. Harper *et al.* determined that heating of VACV at 55°C for one hour damaged the capping enzyme that is required for transcription termination (Harper et al, 1978). These authors showed that RNA transcripts synthesized *in vitro* by heat-treated virions were longer, suggesting a defect in termination of transcription, whilst also being uncapped and partially double-stranded (Harper et al, 1978). The authors proposed that these uncapped and aberrant mRNA were unlikely to be translated into proteins (Harper et al, 1978), which is in agreement with the present finding. Additionally they also could not detect mRNA transcription in cells infected with VACV heated at 55°C, possibly because the uncapped mRNA was degraded too fast. Also, the mRNA transcription was checked

one hour p.i in the current study, assuming that perhaps the mRNA were degraded too fast. Moreover, at that time it was not possible to detect viral mRNA. On the other hand, Cao *et al.* detected that GFP expression under an early viral promoter was significantly reduced in human plasmacytoid dendritic cells infected with heated-VACV-GFP at 55°C for one hour (Cao et al, 2012). Similarly, in the current study it was confirmed that early viral protein expression was completely abrogated in VACV-infected cells, when the virus was heated at 55°C for 1 hr or at 75°C for 20 min. Otherwise, Dai *et al.* proposed that heated-VACV (55°C, 1 hr) induces cytokine production in murine keratinocytes, and that this induction is dependent on sensing cytosolic viral dsRNA (Deng et al, 2008). Similarly, Cao *et al.* demonstrated that VACV heated at 55°C for one hour induced IFN-I production in pDCs which requires activation of endosomal TLR7 and its adaptor MyD88 (Cao et al, 2012). In contrast, Drillien *et al.* suggested that binding or uptake of heated-VACV (55°C) was sufficient to activate DC (Drillien et al, 2004).

VACV WR only induced cytokine production in BMDM, when the virus was heated at 55°C for one hour. This observation coincides with those of Cao *et al.,* who found that VACV heated to 55°C for one hour induces pDCs to produce IFN-α and TNF-α (Cao et al, 2012). In another study it was shown that after incubating VACV at 55°C for 1 hr the virus was still capable of activating human monocyte-derived conventional DC (Drillien et al, 2004). However, when MVA was treated at 75°C for 20 minutes the cytokine production in THP-1 cells and BMDM was completely abolished. In agreement, it has been reported that heating MVA at 75°C for 20 minutes prevented CCL2 protein secretion (Lehmann et al, 2009). Additionally, heating VACV at 65°C for one hour or 100°C for 5 minutes abolished cytokine induction (Cao et al, 2012). Also differences in the virus preparation it would

modulate the sensitivity of the virus to the heating process, fact that influences the results obtained for the differents groups

Taken together, these findings indicate that in MVA-infected macrophages cytokine induction is independent of early viral protein synthesis, viral DNA replication and viral intermediate/late mRNA transcription. Thus, any viral component, maybe early viral mRNA synthesis or genomic DNA, during the early phase of the virus replication cycle, is sufficient to induce cytokine production in MVA-infected macrophages. Similarly, Lynch *et al.* concluded that early events in the viral replication cycle, prior to early protein synthesis and DNA replication were sufficient to promote IκBα degradation in MVA-infected cells (Lynch et al, 2009). Furthermore, the same study showed that MVA activates PKR-mediated signaling pathways even in the absence of DNA replication, suggesting that early events may constitute the first stimuli to PKR activation. Indeed, they and others have demonstrated that viral dsRNA from early transcripts possess PKR activating function (Lynch et al, 2009; Willis et al, 2011). Particularly, with respect to viral mRNA, several studies reported the importance of RNA sensors for detecting VACV infection (Delaloye et al, 2009; Deng et al, 2008; Guerra et al, 2007).

Curiously, when early viral protein synthesis is inhibited in VACV Wyeth or VACV WR, the virus gained the capability to induce cytokine production. This fact suggests that VACV possess the intrinsic capacity to trigger cytokine secretion, which is blocked by immunomodulatory proteins expressed by the virus. Many of these viral proteins that are capable of blocking cellular signaling pathways are expressed during the early phase of the viral replication cycle. Interestingly, when the expression of early viral proteins in MVA is affected, the induction of cytokines is enhanced, suggesting that MVA still carries some genes that are able to inhibit

cellular activation. Therefore, in theory, it should be possible to further improve the immune stimulatory capacity of MVA. Alternatively, in the absence of early protein synthesis there is a continuous early viral mRNA transcription that may induce cytokine expression more potently than in normal conditions.

Previously Lehmann *et al.* have shown that MVA triggered the migration of leukocytes into the lungs of infected mice, in contrast to other VACV strains (Lehmann et al, 2009). MVA upregulated the expression of CCL2, CCL3, CXCL8 and CXCL10 in an intranasal infection model of VACV, and it was demonstrated that CCL2 plays a key role in the early immigration of leukocytes into the lung (Lehmann et al, 2009). Moreover, it was found that MVA induces the expression of the chemotactic factor CCL2 in THP-1 cells. Similar results were obtained in human macrophages, in which MVA induced the production of large quantities of chemokines (CCL2, CCL4, CCL5, CXCL8 and CXCL10) (Delaloye et al, 2009) while only small amounts of pro-inflammatory cytokines such as: TNF-α, IL-1α, IL-1β and IL-6 (Delaloye et al, 2009).

It has been shown that IFNAR stimulation leads to the induction of CCL2 expression (Conrady et al, 2013) Thus, the contribution of IFNAR to MVA-induced chemokine expression in macrophages was investigated. The infection of BMDM from IFNAR$^{-/-}$ mice with MVA demonstrated that signaling through IFNAR plays a crucial role in the induction of CCL2 expression in murine macrophages. Previously it has been reported that systemic release of IFN during MCMV infection drives expression of CCL2 in bone marrow cells, which induces monocyte migration to the site of infection (Crane et al, 2009). Also, the relevance of IFNs for mediating migration and cytotoxic activity of NK cells which are important for controlling MCMV infection has been reported (Orange & Biron, 1996). In a murine infection model of MCMV, IFN-α/β

promoted CCL2 production and plays a distinctive role in the recruitment of macrophages (Hokeness et al, 2005). Moreover, IFN signaling has been linked to VACV specific CD8+ lymphocyte expansion and activation (Quigley et al, 2008). Additionally in the current study it was found that IFN-β mRNA synthesis in MVA-infected $IFNAR^{-/-}$ BMDM was lower than in wild type BMDM, suggesting that the positive feedback loop via IFNAR was relevant for the expression of IFN-β, which is in line with a previous report concerning IFN-α production in pBMDC (Waibler et al, 2007).

Inflammatory cytokine and type I IFN expression is partially mediated *via* the activation of the transcription factor NF-κB, which regulates the promoters of many of these genes (Sung et al, 2002). The activity of NF-κB is modulated by O-GlcNAcylation, and it has been shown that this posttranslational modification of NF-kB is required for full activation of some cells of the immune system (Golks & Guerini, 2008). Thus this study investigated whether O-GlcNAcylation is important for triggering CCL2 expression in MVA-infected macrophages. Firstly it was found that the O-GlcNAcylation pattern of intracellular proteins in MVA-infected THP-1 cells is different compared to non-infected cells, suggesting that MVA infection alters the normal O-GlcNAcylation levels of the host cells. The O-GlcNAcylation pattern of intracellular proteins in MVA-infected THP-1 cells was similar to VACV WR-infected THP-1 cells. Western Blot alone may not be sensitive enough to detect O-GlcNAc modification on specific proteins. Therefore, future studies are necessary to determine whether there are differences in the O-GlcNAcylation pattern of specific intracellular signalling proteins in MVA or VACV-infected cells.

Blocking O-GlcNacylation with alloxan leads to decreased expression of CCL2 in MVA-infected THP-1 cells. This suggests that O-GlcNacylation is necessary for MVA triggered cytokine expression in monocytes, perhaps due to the O-GcNAc

modification of key transcriptions factors like NF-κB. In line with these results, it was reported that chemokine production was regulated by O-GlcNAcylation of nuclear proteins in a monocytic cell line (Chikanishi et al, 2010). Previously, it was shown that full activation of T and B lymphocytes mediated by TCR and BCR, respectively, requires O-GlcNAcylation of NF-κB (Golks et al, 2007). Moreover, O-GlcNAcylation seems to modulate chemotaxis of monocytes induced by MVA-infected THP-1 cells, since CCL2 is one of the main chemotactic factor secreted by MVA-infected monocytes.

6. SUMMARY

The highly attenuated poxvirus strain MVA is a very promising viral vector vaccine candidate against a broad spectrum of infectious diseases. Despite the lack of productive viral replication in most mammalian cells, MVA is highly immunogenic. The mechanism how MVA triggers the innate immune response is not well understood. Thus, the first objective of this study was to investigate which components of VACV play major roles in triggering cellular signaling pathways, leading to the expression of inflammatory cytokines in monocytes/macrophages. It was shown that MVA-induced cytokine expression was independent of viral DNA replication and early viral protein synthesis in human monocytic THP-1 cells. Treatment of MVA with a dosage of UV light that allows transcription of early but not intermediate or late viral RNA did not affect its capability to induce interferon-β (IFN-β) and chemokine expression in THP-1 cells and bone marrow-derived macrophages (BMDM). In contrast to other VACVs, MVA induces cytokine expression in infected macrophages; however when the replication competent VACV strains Wyeth and Western Reserve (WR) were treated with low doses (0.1-0.8 J) of UV light they also gained the capability to induce cytokine production in THP-1 cells and BMDM. Using BMDM deficient for Toll-like receptors 2 (TLR2) and TLR4 it was shown that these receptors play no role in MVA induced cytokine expression. Overall, these results indicate that viral components sensed during an early phase of the viral replication cycle, most likely early viral mRNAs and/or genomic DNAs are sufficient to trigger cytokine expression in MVA-infected macrophages.

To further explore the mechanisms of chemokine induction in MVA-infected BMDM, the involvement of type I interferon receptor (IFNAR) in the modulation of chemokine

(C-C motif) ligand 2 (CCL2) expression was tested. The results indicate that CCL2 expression in MVA-infected BMDM is induced in an IFNAR-dependent manner.

Furthermore, the importance of posttranslational modification by O-GlcNacylation in MVA induced chemokine expression was investigated. O-GlcNacylation of intracellular proteins in MVA-infected THP-1 cells was different as compared to non-infected cells, suggesting that MVA infection alters the normal O-GlcNacylation levels of the host cells. Additionally, it was shown that blocking O-GlcNacylation decreases the expression of CCL2 in MVA-infected THP-1 cells. This data suggests that MVA-induced O-GlcNacylation is relevant for MVA-triggered CCL2 expression.

7. ZUSAMMENFASSUNG

Das hoch attenuierte Modifizierte Vaccinia Virus Ankara (MVA) ist ein vielversprechender Virusvektor-Impfstoffkandidat gegen ein breites Spektrum von Infektionskrankheiten. Obwohl sich MVA in den meisten Säugetierzellen nicht produktiv vermehrt, ist es hoch immunogen. Bisher ist wenig zu den Mechanismen bei der Auslösung einer angeborenen Immunantwort nach MVA-Infektion bekannt. Ein Ziel dieser Studie war es herauszufinden, welche viralen Komponenten zur primären Stimulierung zellulärer Signalkaskaden beitragen und somit zur Expression inflammatorischer Zytokine in Monozyten bzw. Makrophagen führen. Für humane monozytäre THP-1-Zellen wurde gezeigt, dass die MVA-induzierte Zytokinexpression unabhängig von der viralen DNA-Replikation und der Synthese früher viraler Proteine ist. Die Fähigkeit von THP-1-Zellen und Knochenmarksmakrophagen zur Interferon-β (IFN-β)- und Chemokinexpression wurde durch eine UV-Behandlung von MVA mit Strahlungsdosen, die nur noch die Transkription früher, viraler RNA erlaubt, nicht beeinträchtigt. Im Gegensatz zu anderen Vacciniaviren induziert MVA die Zytokinexpression in infizierten Makrophagen. Jedoch führte auch die Behandlung der replikationsfähigen Vacciniavirusstämme Wyeth und Western Reserve (WR) mit niedrigen UV-Strahlungsdosen (0.1-0.8 J) zur Zytokinproduktionsinduktion in THP-1-Zellen und Kochenmarksmakrophagen. Die Toll-Like-Rezeptoren (TLR)-2 und -4 spielen keine Rolle bei der MVA-induzierten Zytokinexpression, was nach der Infektion von Knochenmarksmakrophagen, die diese Rezeptoren nicht aufwiesen, gezeigt werden konnte.

Insgesamt deuten diese Ergebnisse darauf hin, dass bereits erste virale Signale im Replikationszyclus der Viren, höchstwahrscheinlich frühe virale mRNA und/ oder genomische DNA, für die Auslösung der Zytokinexpression in MVA-infizierten

Makrophagen ausreichen. Weiter wurde getestet, ob der Typ-1-Interferon-Rezeptor (IFNAR) die Expression des Chemokin (C-C-Motiv) Liganden-2 (CCL2) moduliert. Die Ergebnisse weisen auf eine IFNAR-abhängige Induktion der CCL-2-Expression in MVA-infizierten Knochenmarksmakrophagen hin. Schließlich wurde auch die Relevanz der posttranslationalen *O*-GlcNAc-Modifikation bei der MVA-induzierten Chemokinexpression untersucht. Die *O*-GlcNAc-Modifikation von intrazellulären Proteinen in MVA-infizierten THP-1-Zellen unterschied sich von der in nicht-infizierten Zellen, was eine Veränderung der normalen *O*-GlcNAc-Modifikation in der Wirtszelle durch eine MVA-Infektion annehmen lässt. Zusätzlich wurde gezeigt, dass die CCL2-Expression in MVA-infizierten THP-1-Zellen über eine Hemmung der *O*-GlcNAc-Modifikation reduziert werden kann. Diese Ergebnisse sprechen für die Relevanz der MVA-induzierten *O*-GlcNAc-Modifikation bei der durch MVA ausgelösten CCL2-Expression.

8. ABBREVIATIONS

β-ME	β-mercaptoethanol
ADAR	adenosine deaminase acting on RNA
Amp	ampicillin
ANO	adenosine N1-oxide
APS	ammonium persulfate
APC	antigen presenting cells
AraC	cytosine arabinoside
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
BMDM	bone marrow-derived macrophages
BM-pDC	bone marrow plasmacytoid dendritic cells
CCL2	chemokine (C-C motif) ligand 2
cDNA	complementary DNA
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CEF	chicken embryo fibroblast
CEV	cell associated enveloped virus
cGAS	DNA sensor cyclic GMP-AMP synthase
CMV	cytomegalovirus
CTL	CD8 cytotoxic lymphocyte
CVA	chorioallantois vaccinia virus Ankara
CXCL8	chemokine (C-X-C motif) ligand 8
CXCL10	chemokine (C-X-C motif) ligand 10
DC	dendritic cells
DMEM	dulbecco`s Modified Eagle Medium
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotide triphosphate
dsDNA	double stranded DNA
DTT	dithiotreitol
ECTV	ectromelia virus

EEV	extracellular enveloped viron
ER	endoplasmic reticulum
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EF1α	elongation factor 1α
FACS	fluorescence activated cell sorting
FCS	fetal calf sera
GAGs	glycosaminoglycans
GAPDH	glycerinaldehyd-3-Phosphat-dehydrogenase
GFP	green fluorescent protein
GlcN	glucosamine
GM-CSF	granulocyte Macrophage colony stimulating factor
HeLa	cervix carcinoma cell line isolated from *Henrietta Lacks*
HRP	horseradish peroxidase
HSV	herpes simplex virus
IEV	intracellular enveloped virus
IFNAR	type I IFN receptor
IFN-I	type I interferon
IFN-β	interferon-β
IMV	intracellular mature viron
ISG	IFN stimulated genes
IgG	immunoglobulin G
IL	interleukin
LPS	lipopolysaccharides
MAVS	mitochondrial antiviral signaling proteins
MCMV	murine cytomegalovirus
MDA5	melanoma differentiation associated gene 5
MHCI	major histocompatibility complex class I
MOI	multiplicitiy of infection
MPV	monkeypox virus
mRNA	messenger RNA
MVA	modified vaccinia virus Ankara
Myd88	myeloid differentiation primary response gene 88
NCOAT	nuclear and cytoplasmic *O*-GlcNAcase and histone

	acetyltransferase
ncOGT	nucleocytoplasmic OGT
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-kappa B
NK	natural killer cells
nt	nucleotide
OAS	2'-5'-oligoadenylate synthetase
OD	optical density
O-GlcNAc	*O*-liked N-Acetyl-D-Glucosamine
O-GlcNAcase	*O*-GlcNAc hexosaminidase
OGT	*O*-GlcNAc transferase
ORF	open reading frames
P_3CSK_4	synthetic lipopeptide *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2R,*S*)-propyl]-(R)-cysteinyl-seryl-(lysyl)3-lysine
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen associated molecular patterns
PKR	protein Kinase R
PMN	polymorphonuclear leukocytes
PRR	Pathogen recognition receptors
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RLR	RIG-I-like receptor
RT	room temperature / reverse transcriptase
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
ssRNA	single stranded RNA
STING	stimulator of interferon genes
TEMED	N, N, N´, N´-tetramethylethylenediamine
TLR	toll-like receptor
TNF-α	tumor necrosis factor alfa
TRIF	TIR-domain-containing adapter-inducing interferon-β
UV	ultraviolet

VACV	vaccinia virus
VACV WR	Western Reserve
VLE	very low endotoxin

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