Modified vaccinia virus Ankara induces Toll-like receptor independent type I interferon responses

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Summary

Modified vaccinia virus Ankara (MVA) is a highly attenuated vaccinia virus strain undergoing clinical evaluation as a replication-deficient vaccine vector against various infections and tumor diseases. To analyze the basis of its high immunogenicity, we investigated the mechanism of how DNA encoded MVA induces type I interferon (IFN) responses. MVA stimulation of bone marrow derived dendritic cells (DC) showed that plasmacytoid DC were main IFN-α producers that were triggered independently of productive infection, viral replication, or intermediate and late viral gene expression. Increased IFN-α levels were induced upon treatment with mildly UV irradiated MVA suggesting that virus encoded immune-modulator(s) interfered with the host cytokine response. Mice devoid of Toll-like receptor (TLR) 9, the receptor for double stranded DNA, mounted normal IFN-α responses upon MVA treatment. Furthermore, mice devoid of adaptors of TLR signaling, MyD88 or TRIF, and mice deficient of protein kinase R (PKR) showed IFN-α responses that were only slightly reduced when compared to wild-type mice. MVA induced IFN-α responses were critically dependent on autocrine/paracrine triggering of the IFN-α/β receptor (IFNAR) and were independent of IFN-β, thus involving “half” a positive feedback-loop. In conclusion, MVA mediated type I IFN secretion was primarily triggered by non-TLR molecules, was independent of virus propagation and critically involved IFN-feedback stimulation. These data provide the basis to further improve MVA as a vaccine vector.
Introduction

Modified vaccinia virus Ankara (MVA) is a highly attenuated virus strain generated by more than 500 passages in chicken embryo fibroblasts (CEF) of a conventional vaccinia virus (VACV) formerly being used as vaccine against human smallpox in Turkey and Germany. During propagation in tissue culture, about 15% of the parental viral genome corresponding to approximately 30 kb DNA got lost. Among the genetic information being lost or inactivated in the MVA genome, there are host range genes, genes involved in virus-host interaction and viral immunomodulatory genes. Probably as a consequence of genomic deletions, MVA is replication-deficient in most cells of mammalian origin. The cascade-like pattern of viral early-, intermediate- and late-gene expression, including DNA replication, is activated in most mammalian cells whereas viral multiplication is arrested at a late stage after accumulation of immature viral particles.

Like many other viruses, the prototype orthopoxvirus VACV evolved strategies to prevent activation of the host immune system or to evade host immune responses. These strategies include expression of viral receptors for certain cytokines such as soluble type I and type II interferons, receptors for TNF and interleukin (IL)-1 and receptors for CC chemokines. In fact, mice deficient for a functional type I IFN and/or type II IFN system showed an increased sensitivity to lethal VACV infection. Moreover, VACV proteins have been identified that specifically target Toll-like receptor (TLR) signaling by associating with the TIR-domain containing adaptor molecules MyD88 and TRIF. It has been shown that viral immunomodulatory proteins encoded by VACV contribute to virulence and it is discussed that the avirulence of MVA, at least in part, is related with the loss of many of the above summarized viral escape mechanisms. Although MVA is highly attenuated and replication deficient, it is surprisingly immunogenic when compared to conventional VACV. Thus, MVA vaccination can induce protective immunity against veterinary orthopoxvirus infections and treatment with MVA enhanced resistance against HSV-1 in mice 100-fold. Considering these properties, recombinant MVA is a promising animal and human vaccine candidate. Since MVA is also regarded as a potential vector for tumor vaccination or vaccination of immunocompromised patients it is of major interest to gain more insight into the basis of MVA-related immunogenicity. In particular type I interferons (IFNs) and their main producers, dendritic cells (DC), play a key role in many vaccination strategies. It has been revealed that MVA drives the immune system towards increased
production of pro-inflammatory cytokines such as type I IFNs and human DC were identified as being moderately activated by infection with MVA.

Upon many viral infections, plasmacytoid dendritic cells (pDC) are important type I IFN producers that are responsible for systemic type I IFN responses in vivo (or see for review). pDC constitute a subset of dendritic cells that is found in humans and mice. Compared to murine myeloid DC (mDC), mouse pDC express CD11c, B220, CD45RB and show reduced CD11b levels (for review see). pDC can be found in many tissues, including secondary lymphoid tissues, liver and lung at low percentages. Using different viral model systems it became evident that pDC may be activated directly by both, RNA-encoded and DNA-encoded viruses. In doing so, TLRs turned out to be crucial sensors of virus associated molecular patterns such as double-stranded (ds) DNA or ds or single-stranded (ss) RNA. Among 12 mammalian TLRs discovered so far, several of them are involved in the recognition of viral components. By triggering TLR9, the DNA genomes of HSV-1 and HSV-2 were identified as the pathogen associated molecular pattern primarily mediating activation of, and cytokine release by, dendritic cells in vivo and in vitro. Additionally, in the case of another herpesvirus, DNA-encoded MCMV, TLR9-dependent recognition by pDC has been reported to be the main trigger of type I IFN. Which of these mechanisms apply for DNA-encoded MVA has not been investigated, yet. In addition, cytosolic non-TLR sensors for dsRNA such as Protein kinase R (PKR), melanoma differentiation-associated gene-5 (mda-5), or retinoic-acid-inducible gene-I (RIG-I) have been identified that play a role in activation of DC. PKR is a cytosolic serine/threonine kinase activated by autophosphorylation on binding to dsRNA that has been implicated in the induction of IFN responses to some viruses. RIG-I seems to be an essential sensor for infection with RNA encoded viruses of fibroblasts and conventional DC and mda-5 is involved in the induction of antiviral cytokines upon infection with influenza-A virus, measles virus, and picornavirus. Moreover, novel cytosolic sensor(s) for dsDNA have recently been reported to activate type I IFN responses in a TLR9 independent and IRF3 dependent, or in a TLR and RIG-I independent manner.

As so far, only little is known how MVA induces strong immune responses, in this study we addressed MVA triggered type I IFN responses by dendritic cell subsets. We found that pDC were strong producers of type I IFN that were induced primarily independently of TLRs.
Moreover, we gained insight in viral and cellular mechanisms that are critically involved in MVA mediated activation of DC.
Materials and Methods

Mice and Viruses

TLR9 deficient mice (TLR9<sup>−/−</sup>) and MyD88-deficient mice (MyD88<sup>−/−</sup>) were provided by Shizuo Akira<sup>1,25</sup>, TRIF deficient mice (TRIF<sup>−/−</sup>) by Bruce Beutler<sup>28</sup>, PKR deficient mice (PKR<sup>−/−</sup>) by Charles Weissmann<sup>53</sup>, and IFN-β (IFN-β<sup>−/−</sup>) deficient mice by Siegfried Weiß<sup>20</sup>. For breeding of MyD88/TRIF double knockout mice, animals obtained drinking water supplemented with the antibiotics sulfamethoxazole (400 µg/ml) and trimethoprim (80 µg/ml) for two weeks of every four. All mice have been backcrossed at least 10x on the C57BL/6 background except PKR<sup>−/−</sup> which were on the SV129 background. Type I interferon receptor deficient mice (IFNAR<sup>−/−</sup>)<sup>40</sup> have been backcrossed 20x on the C57BL/6 background. All mice were bred under specific pathogen free (SPF) conditions at the Zentrale Tierhaltung of the Paul-Ehrlich-Institut. Unmutated C57BL/6 and SV129 mice were purchased from Charles River. Mouse experimental work was carried out using 8- to 12-week old mice in compliance with regulations of German animal welfare.

Modified vaccinia virus Ankara (MVA) (cloned isolate F6 at 584<sup>nd</sup> CEF passage) was routinely propagated and titrated on chicken embryo fibroblasts (CEF). For analysis of productive infection, BM-derived Flt3-L or GM-CSF cultures were infected with MVA at MOI 0.1 and cells harvested after 1 h of viral adhesion (0 h). Other aliquots were washed after 1 h of viral adhesion and incubated with fresh medium. 48 h later these supernatants plus BM-DC were harvested (48 h). Supernatants plus cells of each time point were freeze/thawed 3 times, sonicated and back-titrated in serial dilutions on CEF. MVA specific plaques were stained using a purified polyclonal anti-vaccinia immune globulin (VIG) prepared from serum of vaccinated humans. As a control, CEF were infected with the original MVA inoculum at MOI 0.05 and 0 h and 48 h later virus was determined as described above.

For UV irradiation of virus a UV irradiation chamber (Herolab) was used. In such a device, UV irradiation is adjusted by energy per area (in mJ/cm²). A typical 300 mJ/cm² irradiation usually took approximately 45 seconds.

Cell isolation and culture

BM cells were isolated by flushing femur and tibia with RPMI supplemented with 10% FCS. Upon red blood cell lysis, cells were washed and seeded at a density of 1x10⁶ cells/ml or 2x10⁶ cells/ml in medium supplemented with GM-CSF (100 ng/ml; R&D Systems) or Flt3-L (100 ng/ml; R&D systems), respectively. Flt3-L supplemented cultures (BM-pDC) were
cultivated for 8 days with one medium change at day four, whereas medium of GM-CSF supplemented cultures (BM-mDC) was changed every one to two days, depending on the status of cultures, by replacing half of the medium with fresh cytokine-supplemented medium.

Flow cytometric analysis and cell enrichment
Cells were stained with anti-B220-PE or -PECy5.5 mAb, anti-CD11c-APC mAb, anti-CD69-PE mAb, or anti-CD86-FITC mAb (all from BD PharMingen). For detection of vaccinia specific surface proteins VIG was used in combination with Fab(2)-PE. For enrichment of CD11c⁺B220⁺ cells from Flt3-L BM-cultures MS or LD columns (Miltenyi Biotech) were used according to manufacturer’s instructions. The purity of pDC (CD11c⁺B220⁺) usually exceeded 80%.

In vitro stimulations and quantification of cytokine production
For stimulation experiments, ex vivo isolated BM cells or in vitro differentiated bulk cultures of BM-mDC and bulk cultures of BM-pDC were seeded at 1x10⁶ cells/well in 24-well culture plates in 1 ml medium. MACS-sorted cells were seeded at a density of 2x10⁵ cells/well in 96-well culture plates in 200 µl medium. CpG 2216 (ggGGGACGATCGTCgggggG; Sigma-ARK) was used at a final concentration of 10 µg/ml. For transfection of 2 µg pI:C (Sigma-Aldrich), the reagent Fugene (Roche) was used according to manufacturer’s instructions. After stimulation, cell-free supernatant was collected and analyzed with a mouse IFN-α or mouse IFN-β ELISA kit (PBL Biomedical Laboratories).
Results

*MVA but not VACV induces type I interferon responses upon immunization of mice and infection of dendritic cells*

Previously it has been reported that MVA induces Flt3-L mediated expansion of dendritic cells (DC) in newborn mice, a process in which interferons (IFNs) were postulated to play a crucial role. However, it is still unclear whether MVA is able to induce systemic type I IFN responses in vivo. Here we show that 12 hours after i.v. inoculation of wild type (WT) mice with $1 \times 10^7$ pfu MVA, serum IFN-α level peaked at about 350 pg/ml, declined within the next 6 hours and reached background levels 24 hours after infection (Fig. 1A, black squares). In stark contrast, upon inoculation of mice with $1 \times 10^5$ pfu vaccinia virus (VACV) no IFN-α was detected in the serum of mice (Fig. 1A, open circles). When $1 \times 10^7$ pfu VACV were used for immunization no IFN-α was measurable either (data not shown). Thus, unlike VACV, MVA is able to induce systemic type I IFN responses in mice.

When total bone marrow (BM)-cells were infected with MVA at different multiplicities of infection (MOIs) IFN-α secretion was elicited with the highest levels reached at MOI 1 (Fig. 1B, black bars). To gain more insight into which cell type primarily produced IFN-α, BM-derived Flt3-L expanded plasmacytoid (BM-pDC) and GM-CSF expanded myeloid (BM-mDC) dendritic cell cultures were generated. Upon MVA infection, BM-pDC secreted high IFN-α levels, whereas BM-mDC produced lower IFN-α quantities (Fig. 1C and 1D, black bars). Reminiscent of the in vivo observations, VACV was not able to induce IFN-α responses in vitro (Fig. 1B, 1C, and 1D; white bars). As a positive control, the Toll-like receptor (TLR) 9 ligand double stranded CpG containing oligonucleotide 2216 was used to elicit IFN responses by pDC but not by mDC (Fig. 1C and 1D).

We also analyzed pDC sorted by MACS technique from BM-pDC cultures for type I IFN expression upon MVA infection. These experiments confirmed that pDC were important producers of IFN-α (Fig. 2A). Furthermore, within BM-DC cultures B220$^+$CD11c$^+$ pDC and B220$^+$CD11$^+$ mDC upregulated activation markers CD69 and CD86 upon MVA infection (Fig. 2B). Thus, we showed that in contrast to VACV, MVA was able to elicit IFN responses in vivo, and in vitro infection experiments revealed that MVA induced pDC to produce high amounts of IFN-α.
MVA mediated IFN-α induction in dendritic cells is independent of productive infection, viral replication, or intermediate and late viral gene expression

To further explore conditions required for the induction of type I IFN, we investigated whether productive infection took place upon MVA stimulation of BM-DC. To this end supernatants of MVA infected BM-DC cultures were back-titrated on chicken embryo fibroblasts (CEF). These experiments revealed no evidence for MVA propagation in BM-DC within 48 hours after infection (Fig. 3A). Thus, a productive viral infection was not required for the induction of IFN-α in BM-DC. As a positive control CEF were infected with the original MVA inoculum that indeed showed productive infection of the cells (Fig. 3A, gray circles). Interestingly, MVA irradiated with low dosages of UV light induced enhanced IFN-α responses by BM-pDC and BM-mDC (Fig. 3B). This further indicated that induction of IFN-α was independent of newly synthesized viral gene products and that MVA might still encode for viral inhibitors interfering with host immune response. To further determine the relevance of the viral life cycle for the induction of IFN-α, DC were infected with MVA in the presence of cytosine β-D-arabinofuranoside (AraC) that inhibits DNA replication and suppresses expression of intermediate and late viral genes. These data showed that AraC had no influence on the secretion of IFN-α by MVA infected BM-pDC (Fig. 3C; black bars). Interestingly, when BM-mDC were MVA infected in the presence of AraC, a slight reduction of IFN-α secretion was observed (Fig. 3C; white bars). To test whether viral proteins were synthesized by MVA infected BM-DC, untreated (gray shaded curves) and MVA infected (black curves) cells were stained with a purified polyclonal anti-vaccinia immune globulin 18 hours after infection (Fig. 3D; for details refer to materials and methods section). MVA infection led to expression of viral proteins on the surface of both BM-DC-subsets. Thus, induction of type I IFN upon MVA infection of BM-DC is independent of productive infection and does not involve viral DNA replication and expression of intermediate or late viral gene products.

Sensing MVA is largely independent of TLRs and PKR

We next wanted to gain insight into cellular mechanisms of sensing MVA infection. Therefore, we generated BM-pDC from WT mice and mice deficient for components of TLR signaling, including TLR9, MyD88, or/and TRIF, and mice that were deficient for PKR. The total number and the phenotype of BM-pDC derived from the different knockout mice were comparable with that of WT mice (data not shown). BM-pDC were infected with MVA at the MOI 1, or they were treated with 10 μg/ml CpG, or 2 μg transfected pl:C, or they were left untreated as controls. As shown in Fig. 4A, MVA induced IFN-α responses in TLR9 deficient
BM-pDC as good as in WT cells, indicating that triggering of TLR9 by the DNA genome of MVA was not the relevant mechanism responsible for the induction of IFN-α in BM-pDC. Interestingly, upon MVA stimulation of BM-pDC deficient for MyD88, which is one main adaptor molecule mediating signaling by TLR1, TLR2 and TLR4-11, a reduced IFN-α secretion was observed when compared to WT BM-pDC (Fig. 4A).

Double stranded RNA (dsRNA) is an intermediate of viral replication that has been implicated in type I IFN induction upon viral infections \(^{39,42}\). Among TLRs identified so far, TLR3 is involved in sensing dsRNA and to use TRIF as an adaptor for signal transduction (reviewed in 3). In line with this, TRIF\(^{-/-}\) BM-pDC transfected with pI:C showed reduced IFN-α responses when compared to WT controls (Fig. 4A). Nevertheless, MVA infection of TRIF deficient BM-pDC resulted in similar IFN-α production as observed upon infection of WT BM-pDC. Protein kinase R (PKR) deficient BM-pDC infected with MVA showed IFN-α levels comparable to those observed in WT control cells (Fig. 4A) indicating that this pathway did not significantly participate in MVA mediated IFN-α induction. Since PKR deficient mice were on the SV129 background, SV129 WT controls were used that showed results comparable to WT controls from the C57BL/6 background (data not shown). To gain detailed insight into the role of TLR signaling in the induction of IFN-α responses upon MVA infection, we generated BM-pDC from mice which were deficient for both TLR adaptor molecules MyD88 and TRIF. Infection of MyD88/TRIF double knockout BM-pDC with different MOI MVA resulted in IFN responses that were reduced when compared to WT controls (Fig. 4B). Thus, complete loss of TLR signaling in BM-pDC, diminished but did not abrogate IFN-α production upon MVA infection.

It is well accepted that sensing of viruses or artificial stimuli that trigger TLRs located within the endosomes, critically involves endosomal acidification \(^{16,36}\). Since we observed a reduction of IFN-α secretion when MyD88 deficient or MyD88/TRIF double knockout BM-pDC were MVA infected (Fig. 4A and 4B), we aimed to determine whether sensing of MVA and subsequent IFN-α secretion required endosomal acidification. Therefore, BM-pDC were treated with the virus in presence or absence of chloroquine, a lysomotropic agent preventing endosomal acidification. Chloroquine completely abrogated CpG ODN induced IFN-α production that is triggered by TLR9 in the endosomes and critically requires endosomal acidification (Fig. 4A, 4B, 4C, and \(^{16,36}\)). The inhibition was not related with drug toxicity, because chloroquine had no measurable effect on BM-DC viability (data not shown and \(^{16}\)). However, upon MVA infection in the presence of chloroquine, IFN-α responses by BM-pDC were reduced by approximately 40% (Fig. 4C). Taken together these data suggested that
MVA induced IFN-α production by BM-pDC did not involve sensing of viral replication via the dsRNA binding protein PKR. Furthermore, absence of the TLR adaptor molecule TRIF did not influence IFN-α levels secreted by BM-pDC in vitro. Although DNA sensor TLR9 was not crucially involved in the recognition of MVA resulting in IFN-α secretion by BM-pDC, the TLR adaptor MyD88 seemed to participate in MVA sensing to some extent. Furthermore, MVA recognition by BM-pDC partially involved endosomal acidification.

To investigate the in vivo impact of the data obtained in vitro with BM-pDC, mice deficient for the receptors or signaling components tested above were used. Upon MVA infection of wild type (WT) control mice, serum IFN-α levels peaked 12 hours after challenge with about 300 pg/ml, declined within the next 6 hours and reached background levels 24 hours after infection (Fig. 4D). In line with the results obtained by infection of BM-pDC in vitro, deficiency for TLR9 had no effect on serum IFN-α levels observed upon MVA challenge of mice. Furthermore, TRIF, MyD88, and PKR deficient animals mounted IFN-α responses that were only slightly reduced compared to WT controls (Fig. 4D). Thus, results obtained by MVA infection of TLR9, MyD88, TRIF, and PKR deficient mice confirmed in vitro studies performed with BM-pDC.

**IFNAR but not IFN-β is critically involved in IFN-α secretion upon MVA infection**

The role of positive feedback for the induction of IFN-α has been discussed controversially. Particularly for pDC a feedback-independent pathway involving TLRs, IRAK4, TRAF6, and IRF7 has been suggested. To determine the role of positive feedback, WT, IFN-β deficient and type I IFN receptor (IFNAR) deficient BM-pDC were MVA infected. Number and phenotype of BM-pDC generated from knockout mice were comparable to that of WT mice (data not shown). As a control, BM-pDC were stimulated with CpG or pI:C or they were left untreated. As shown in Fig. 5A, BM-pDC from IFN-β−/− mice mounted IFN-α levels comparable to that from WT animals. In stark contrast, IFNAR deficient cells showed complete abrogation of IFN-α responses upon MVA infection or treatment with CpG or pI:C. The inability to produce IFN-α was not related with an overall impairment of cells, since production of other cytokines such as IL-12p40 was not altered upon CpG stimulation (data not shown). In line with that, IFN-β but not IFNAR deficient mice challenged with MVA mounted IFN-α responses comparable to those observed in WT animals. A peak of IFN-α production 12 hours after infection was detected in WT and IFN-β deficient mice (Fig. 5B).
Thus, secretion of IFN-α upon in vitro MVA infection of BM-pDC and in vivo challenge was independent of IFN-β but critically involved IFNAR feedback.
Discussion

MVA was used in large field trials in the 1970ies for vaccination against smallpox with no significant side effects recorded. Considering safety, immunogenicity, and the inability to replicate in most mammalian cells, MVA is an ideal vaccine vector candidate. However, despite the usage of MVA in many vaccination studies, little is known about cellular and viral mechanisms contributing to its high immunogenicity. In this study we provide first evidence that MVA induces systemic type I IFN responses in vivo that are contributed by plasmacytoid DC (Fig. 1 and 2) largely independent of TLR and PKR signaling (Fig. 4). We showed that MVA mediated IFN-α induction in dendritic cells was independent of productive infection, viral replication, or intermediate and late viral gene expression (Fig. 3). Furthermore, the data presented here indicate that IFNAR, but not IFN-β, is critically required for MVA-mediated IFN-α induction (Fig. 5).

MVA lacks several immunomodulatory proteins encoded by many other orthopoxviruses as well as host range genes and other genes involved in virus-host interaction. MVA was found to induce NF-κB activation in human embryonic kidney cells and to activate human DC via a NF-κB dependent mechanism. VACV A52R protein, which ORF was deleted during MVA attenuation, can block activation of NF-κB by multiple TLRs by association with IRAK2 and TRAF6. Furthermore, VACV protein A46R targets TLR adaptors (including MyD88 and TRIF), a process contributing to virulence. These findings indicated that targeting and/or antagonizing TLR pathway components might be important mechanisms to escape from host antiviral defense upon VACV infection. Thus, it was likely that sensing of MVA involved components of the TLR pathway. We showed here that in contrast to other DNA-encoded viruses like HSV or MCMV TLR9 was not involved in the induction of type I IFN responses upon MVA infection (Fig. 4A and 4D). However, upon infection of MyD88 deficient BM-pDC or mice, observed IFN-α levels were slightly reduced when compared to WT controls (Fig. 4A and 4D). Reduced IFN levels induced upon MVA infection of BM-pDC were also detected when MyD88/TRIF double knockout cells were tested (Fig. 4B). Pre-treatment of cells with chloroquine, a lysomotropic agent preventing endosomal acidification and thus proper TLR3, 7/8, and 9 function, reduced IFN-α production about 40% when compared to levels reached in the absence of chloroquine (Fig. 4C). These data suggest that even though MVA induced IFN-α secretion was independent of TLR9 triggering, MyD88 was involved in this process at least to some extent. Interestingly,
very recent findings indicated that TLR2/MyD88 was required for VACV induced secretion of pro-inflammatory cytokines. Our results suggest multiple mechanisms involved in sensing MVA infection by TLR dependent and independent mechanisms. These mechanisms, most probably, engaged the DNA genome of MVA, early viral RNA, and/or some viral protein(s). Since plasmacytoid DC are the major source of IFN-α upon infection with MVA (Fig. 1 and 2), RIG-I might be of minor importance in detection of MVA infection since this molecule is merely involved in the detection of viral infections by fibroblasts and conventional DC, but not pDC. The role of mda-5 and/or the recently discovered jet unidentified cytoplasmic DNA-sensor(s) in the recognition of MVA still needs to be elucidated.

As observed by Drillien et al., activation of human DC (upregulation of activation marker CD86) was not impaired when UV irradiated MVA was used for infection. We showed, that MVA induced IFN-α production by myeloid or plasmacytoid DC cultures was enhanced when mildly UV irradiated virus was used (Fig. 3B). These data suggested that although MVA is highly attenuated, it probably still encodes for one or more genes interfering with the host’s IFN-α responses. Interestingly, when graded MOI of MVA were used for infection of BM cells or DC subsets, the highest IFN levels were elicited at MOI 1 whereas at MOI 10 less type I IFN was induced (Fig. 1 and Fig. 4). Most probably this was due to cytopathic effects observed at infections with high dose (MOI 10) MVA that were not experienced when a ten-fold lower infection dose was used (data not shown). It will be a matter of future investigations whether further deletions in the MVA genome are able to improve or to decrease MVA related immunogenicity. The data presented in this study indicate that infection of DC with MVA did not interfere with their capacity to mount IFN responses (Fig. 1). In contrast to other viruses primarily activating plasmacytoid DC, MVA activated both, plasmacytoid and myeloid DC in terms of upregulation of the activation markers CD69 and CD86 (Fig. 2) and production of IFN-α (Fig. 1). This is in line with the TLR9 independent MVA induced IFN-α responses (Fig. 4), because only pDC but not mDC can be triggered via TLR9 to produce IFN-α (Fig. 1C and 1D).

As aforementioned, there is an ongoing debate about the role of the positive feedback via the interferon-α/β receptor (IFNAR) in production of IFN-α. Particularly for pDC a feedback-independent pathway involving TLRs, IRAK4, TRAF6, and IRF7 was suggested. Our data clearly demonstrate that IFNAR expression is absolutely crucial for IFN-α production in
vivo and in vitro. In the absence of this receptor no IFN-α production was observed upon MVA challenge (Fig. 5A and 5B). In contrast, although MVA induced large amounts of IFN-β upon infection of DC and significant systemic serum levels were found upon in vivo challenge (data not shown), IFN-β expression was no prerequisite for IFN-α secretion as indicated by MVA-induced IFN-α responses by IFN-β deficient cells and mice. Thus, “half” a feedback loop involving IFNAR triggering but not IFN-β was required for production of IFN-α upon MVA infection. Interestingly, the type I IFN system played an essential role in control of a VACV infection. In our experiments both, IFN-β and IFNAR deficient mice survived MVA infections without developing any signs of disease (Fig. 5 and data not shown) further supporting the safety of MVA compared to the parental VACV.

The study presented here gives important and detailed insight into mechanisms involved in systemic type I IFN production upon in vivo challenge with MVA. We showed that DC of the plasmacytoid lineage played a key role in the secretion of IFN-α. We found that sensing of MVA infection and subsequent type I IFN secretion is a multiple step process involving TLRs to a minor degree and primarily non-TLR molecules. Furthermore, our data suggest that although highly attenuated, MVA still encodes for one or several immune modulator(s) interfering with the host antiviral defense. Thus, the presented data shed light on mechanisms conferring immunogenicity of MVA and provide the basis for developing new strategies for further improving MVA related immunogenicity and its capacity as a vaccine vector.

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Figure Legends

Fig. 1: MVA but not VACV induces IFN-α responses in vivo and by plasmacytoid and myeloid dendritic cells. (A) Mice were i.v. inoculated with 1x10^7 pfu MVA (black squares) or 1x10^5 pfu VACV (open circles). Serum was collected at the indicated time points after injection and analyzed for IFN-α by an ELISA method. (B) Total bone marrow (BM) cells, BM-derived pDC cultures (C), or BM-derived mDC cultures (D) were infected with MVA (black bars) or VACV (white bars) at the indicated MOIs. Control cells were treated with CpG 2216 (10 µg/ml) or were left untreated (unstim.). 24 h after stimulation or infection cell-free supernatant was collected and analyzed for IFN-α by an ELISA method. Data shown are representative of two to three independent experiments. *, P <0.05 ≥0.01; **, P <0.01 ≥0.001; ***, P <0.001 by unpaired two-tailed t test. Error bars indicate standard deviations from triplicate ELISA measurements.

Fig. 2: Dendritic cells get activated moderately upon MVA infection. (A) B220^+CD11c^+ pDC were MACS-sorted form Flt3-L BM-pDC cultures and infected with MVA at the indicated MOIs. Control cells were treated with CpG 2216 (10 µg/ml) or were left untreated (unstim.). 24 h after stimulation or infection cell-free supernatant was collected and analyzed for IFN-α by an ELISA method. (B) Flt3-L derived BM-pDC or GM-CSF derived BM-mDC cultures were infected with MVA at the MOI 1. 16 h after infection upregulation of CD86 and CD69 on B220^+CD11c^+ BM-pDC or on B220^−CD11c^+ mDC documented activation of both DC subsets. Data shown are representative of two independent experiments.

Fig. 3: IFN-α induction in BM-DC by MVA does not require productive infection or viral replication. (A) Flt3-L derived BM-pDC (black squares) or GM-CSF derived BM-mDC cultures (open squares) were infected with MVA in the MOI 0.1. 0 h and 48 h after BM-DC infection supernatants plus cells were harvested and back-titrated on CEF revealing no evidence for MVA propagation in both BM-DC subsets. As a control CEF (gray circles) were infected with the original MVA inoculum and 0 h and 48 h values were determined as for BM-DC. (B) Flt3-L derived BM-pDC (black bars) or GM-CSF derived BM-mDC cultures (white bars) were infected with untreated MVA MOI 1 or MVA MOI 1 that was irradiated with UV light at the indicated doses [mJ/cm²] prior to infection. Control cells were left untreated. 24 h after infection cell-free supernatant was collected and analyzed for IFN-α by an ELISA method. (C) Flt3-L derived BM-pDC (black bars) or GM-CSF derived BM-mDC
cultures (white bars) were infected with MVA MOI 1 in the presence or absence of AraC (40 µg/ml). Control cells were left untreated. 24 h after stimulation or infection cell-free supernatant was collected and analyzed for IFN-α by an ELISA method. (D) BM-DC were infected with MVA MOI 1 (black curves) or were left untreated (gray shaded curves). 18 h after infection cells were stained for virus specific proteins with VIG in combination with Fab(2)-PE indicating that viral proteins are expressed on the surface of both DC subsets. Data shown are representative of two to four independent experiments. ***, P <0.001 by unpaired two-tailed t test. NS, not significant. Error bars indicate standard deviations from triplicate ELISA measurements.

**Fig. 4: Sensing of MVA is largely independent of TLRs or PKR.** (A) Flt3-L derived BM-pDC cultures were generated from WT mice and mice deficient for TLR9, MyD88, TRIF, or PKR. Cells were infected with MVA MOI 1 or were stimulated with CpG 2216 (10 µg/ml) or with pI:C (for transfection of 2 µg pI:C, Fugene reagent was used) or were left untreated. 24 hours after stimulation or infection cell-free supernatant was harvested and analyzed for IFN-α by an ELISA method. (B) Flt3-L derived BM-pDC cultures were generated from WT mice and mice double-deficient for both, MyD88 and TRIF. Cells were infected with MVA at the indicated MOI or were stimulated with CpG 2216 (10 µg/ml) or pI:C (for transfection of 2 µg pI:C Fugene reagent was used). Controls were left untreated (unstim.). 24 hours after stimulation cell-free supernatant was harvested and analyzed for IFN-α by an ELISA method. (C) WT Flt3-L derived BM-pDC cultures were infected with MVA MOI 1. Controls were stimulated with CpG 2216 (10 µg/ml) or were left untreated. Where indicated, cells were pre-incubated with chloroquine (10 µM) for 2 hours. 24 hours after stimulation cell-free supernatant was harvested and analyzed for IFN-α by an ELISA method. (D) Indicated mice were i.v. inoculated with 1x10^7 pfu MVA. Serum was collected at the indicated time points after injection and analyzed for IFN-α by an ELISA method. Data shown are representative of two to four independent experiments. *, P <0.05; **, P <0.01; ***, P <0.001 by unpaired two-tailed t test. Error bars indicate standard deviations from triplicate ELISA measurements. Standard deviation maxima in (D) were ± 22.7 pg/ml.

**Fig. 5: Positive feedback loop via the IFNAR is critical for MVA induced IFN-α production.** (A) Flt3-L derived BM-pDC cultures were generated from WT mice and mice deficient of IFN-β or IFNAR. Cells were infected with MVA MOI 1 or were stimulated with
CpG (40 nM) or pl: C (for transfection of 2 μg pl: C, Fugene reagent was used) or were left untreated. 24 hours after stimulation cell-free supernatant was harvested and analyzed for IFN-α by an ELISA method. (B) Indicated mice were i.v. inoculated with 1x10^7 pfu MVA. Serum was collected at the indicated time points after injection and analyzed for IFN-α by an ELISA method. Data shown are representative of two to four independent experiments. Error bars in (A) indicate standard deviations from triplicate ELISA measurements. Standard deviation maxima in (B) were ± 16.2 pg/ml.
Reference List


strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine 12:1032-1040.


Figure 1
Figure 2
Figure 3
Figure 4
Figure 5