Myristoylation increases the CD8+ T-cell response to a GFP prototype antigen delivered by modified vaccinia virus Ankara

Lisa Marr, Anna-Theresa Lülf, Astrid Freudenstein, Gerd Sutter and Asisa Volz

German Centre for Infection Research (DZIF), Institute for Infectious Diseases and Zoonoses, LMU University of Munich, Veterinaerstrasse 13, D-80539 Munich, Germany

Abstract

Activation of CD8+ T-cells is an essential part of immune responses elicited by recombinant modified vaccinia virus Ankara (MVA). Strategies to enhance T-cell responses to antigens may be particularly necessary for broadly protective immunization against influenza A virus infections or for candidate vaccines targeting chronic infections and cancer. Here, we tested recombinant MVAs that targeted a model antigen, GFP, to different localizations in infected cells. In vitro characterization demonstrated that GFP accumulated in the nucleus (MVA-nls–GFP), associated with cellular membranes (MVA-myr–GFP) or was equally distributed throughout the cell (MVA–GFP). On vaccination, we found significantly higher levels of GFP-specific CD8+ T-cells in MVA-myr–GFP-vaccinated BALB/c mice than in those immunized with MVA–GFP or MVA-nls–GFP. Thus, myristoyl modification may be a useful strategy to enhance CD8+ T-cell responses to MVA-delivered target antigens.

Introduction

Modified vaccinia virus Ankara (MVA) is a replication-deficient and safety-tested vaccinia virus strain that can be engineered as a vector virus encoding foreign antigens (Sutter & Moss, 1992; Sutter et al., 1994). Today, MVA vectors serve as an established platform technology for developing vaccines against infectious diseases and cancer (Altenburg et al., 2014; Kreijtz et al., 2013; Sebastian & Gilbert, 2016; Volz & Sutter, 2013). Various recombinant MVA have been tested successfully in phase I–IIb clinical trials, and have been found to be safe and immunogenic, inducing both target antigen-specific antibodies and cellular immune responses (Gilbert, 2013; Gómez et al., 2013). In a recent phase I study, immunizations with recombinant MVA delivering the haemagglutinin (HA) antigen of influenza A virus H5N1 (MVA–HA) induced high levels of H5-specific antibodies (Kreijtz et al., 2014).

In addition, the ability to activate strong cellular immune responses is an important factor for the use of recombinant MVA in the search for influenza vaccines with improved efficacy. Enhancing antigen-specific T-cell responses might be a promising strategy for developing broadly protective vaccines against influenza A virus. So far, two major parameters are reported to influence efficient T-cell responses: (i) optimal use of early promoters for recombinant gene expression to support direct antigen presentation and priming of T-cells (Bronte et al., 1997; Kastenmuller et al., 2006, 2007); and (ii) the synthesis and delivery of stable mature protein antigens as preferred substrates for efficient priming of T-cells by cross-presentation (Gasteiger et al., 2007; Pascutti et al., 2011).

Localizing target antigens to subcellular compartments is also considered as an innovative approach to enhance the cellular immune response (Gasteiger et al., 2007). To analyse the effect of different antigen localizations on immunogenicity, we chose GFP as a model antigen and assessed the induction of GFP epitope-specific CD8+ T-cells mediated by recombinant MVA producing either unmodified GFP or GFPs containing nuclear localization (nls) or myristoylation (myr) signals. The gene sequences encoding the target antigens GFP, myr–GFP and nls–GFP were introduced at the site between the vaccinia virus (VACV) G1L and I8R genes (all gene nomenclatures are as established for VACV strain Copenhagen; Goebel et al., 1990) by homologous recombination and placed under the transcriptional control of Pvgf promoter sequences (Fig. 1a). Pvgf is a natural vaccinia virus promoter controlling the abundant early expression of VACV ORF C11R mRNA (Yang et al., 2015).

Recombinant MVA expressing unmodified GFP served as a baseline vaccine. To achieve increased transport of GFP to cellular membranes, we added a myristoylation signal (Chan et al., 2011; Maurer-Stroh et al., 2002), and for nuclear localization we tagged the GFP to a GFP prototype antigen delivered by modified vaccinia virus Ankara (MVA). Strategies to enhance T-cell responses to antigens may be particularly necessary for broadly protective immunization against influenza A virus infections or for candidate vaccines targeting chronic infections and cancer. Here, we tested recombinant MVAs that targeted a model antigen, GFP, to different localizations in infected cells. In vitro characterization demonstrated that GFP accumulated in the nucleus (MVA-nls–GFP), associated with cellular membranes (MVA-myr–GFP) or was equally distributed throughout the cell (MVA–GFP). On vaccination, we found significantly higher levels of GFP-specific CD8+ T-cells in MVA-myr–GFP-vaccinated BALB/c mice than in those immunized with MVA–GFP or MVA-nls–GFP. Thus, myristoyl modification may be a useful strategy to enhance CD8+ T-cell responses to MVA-delivered target antigens.

Two supplementary figures are available with the online Supplementary Material.
recombinant virus MVA-P7.5–GFP was used, encoding GFP under the transcriptional control of the natural VACV early/late promoter P7.5 (Mackett et al., 1982), the first and probably still most widely used promoter for constructing recombinant VACVs.

Our resulting recombinant MVA viruses should equally distribute GFP throughout the cell or deliver GFP predominantly to the cell nucleus (MVA-nls–GFP) or cellular membranes (MVA-myr–GFP) (Fig. 1c). These viruses were purified and quality controlled according to standard

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Fig. 1. Design of the recombinant MVAs. (a) Schematic diagram of the MVA genome containing the six major deletion sites (I–VI). The I8R–G1L site was used to insert GFP, myr–GFP or nls–GFP, under the transcriptional control of the early VACV promoter Pvgf (5′-GTTTATATTACTGAATTAATAATATAAAATTCCCATTGATTCCTGACAA-3′; underlined A indicates transcriptional start site +1 as described by Broyles et al., 1991). (b) Schematic representation of the MVA constructs with the nucleotide and amino acid signal sequences used to construct the different GFP variants. (c) Scheme of GFP localization within infected cells, with GFP represented by grey shading.
procedures for generating recombinant MVA vaccines (Kremer et al., 2012b). During plaque purification, we analysed genetic integrity and stability by PCR using oligonucleotide primers to confirm MVA identity (PCR of the six major deletion sites of MVA in MVA-nls–GFP, MVA-myr–GFP, MVA–GFP and MVA-P7.5–GFP; Fig. S1, available in the online Supplementary Material) and correct insertion of GFP gene sequences within the MVA genome: MVA-nls–GFP, MVA-myr–GFP and MVA–GFP between the G1L and I8R genes, and MVA-P7.5–GFP insertion in deletion site III (Fig. S1).

To analyse growth behaviour, we infected permissive DF-1 and non-permissive MEF and NIH3T3 cells with the recombinant viruses and collected cells and supernatants at the indicated times post-infection (p.i.) (Fig. S2). Titration by immunostaining was performed as described previously (Kremer et al., 2012b). The recombinant MVAs replicated efficiently in the avian cell line (DF-1), but not in cells of mammalian origin (MEF and NIH3T3) (Fig. S2). These findings confirmed the expected MVA phenotype, allowing handling of the recombinant viruses under Biosafety Level 1 conditions.

Next, we assessed the correct expression and predicted cellular localization of the GFPs by immunofluorescence microscopy of MVA–GFP-infected cells (Fig. 2a). Infected DF-1 cells were fixed 24 h p.i. and immunostained as described previously (Boulanger et al., 2002) using primary anti-GFP antibody (Life Technologies) and secondary antibody. The results showed correct expression of the GFPs in the expected cellular localization.

Fig. 2. Characterization of recombinant GFPs. (a) Immunofluorescence staining of DF-1 cells infected with MVA viruses at an m.o.i. of 0.05 on cover slides and fixed at 24 h p.i. Nuclei were stained with DAPI (blue). Fluorescent images were captured with a Keyence BZ-X710 fluorescence microscope at a magnification of ×100. (b) Western blot analysis of cell lysates prepared from DF-1 cells infected with MVA–GFP, MVA-nls–GFP, MVA-myr–GFP and MVA-P7.5–GFP at an m.o.i. of 5, with or without the addition of cytosine arabinoside (AraC), at 24 h p.i.
Alexa Fluor 488-conjugated antibody (Life Technologies). Cell nuclei were stained with DAPI (300 nM). As anticipated, we observed different patterns of green fluorescence, with varying cellular localizations depending on the MVA–GFP construct. Serial optical sections showed that MVA-nls–GFP expressed protein was located predominantly within the cell nucleus. In contrast, MVA-myr–GFP expressed GFP that accumulated primarily on membranous structures, e.g. nuclear and cytoplasmic membranes, but was notably absent within the nucleus. MVA–GFP and MVA-P7.5–GFP rarely showed specific stained areas of green fluorescence, indicating equal distribution of GFP throughout the infected cell.

To further analyse GFP synthesis, total cell lysates from infected CEF cells were analysed by Western blotting using an anti-GFP rabbit antibody (diluted 1 : 250; Life Technologies) and a secondary anti-rabbit antibody (Cell Signalling Technology). We compared GFP expression at 24 h p.i. in DF-1 cells (Fig. 2b). β–Actin antibody (1 : 500; Thermo Scientific) was included to provide a loading control. Similar amounts of GFP were detected for all viruses tested. We also added cytosine arabinoside (AraC, 1 mg ml−1; Thermo Scientific) to the medium to inhibit viral DNA replication and synchronize all infections to strict early gene expression. As expected, the amounts of GFP produced by the control MVA-P7.5–GFP were clearly reduced compared with infection in the absence of AraC. In contrast, the addition of AraC increased the expression of GFP produced by MVA–GFP, MVA-myr–GFP and MVA-nls–GFP because of prolonged activity of viral early transcription.

To assess the immunogenicity of these recombinant MVA–GFP candidate vaccines in vivo, we investigated the activation of GFP-specific CD8+ T-cells in BALB/c mice. Groups of BALB/c mice were inoculated once (at day 1) or twice (at days 1 and 21) intramuscularly with 108 p.f.u. of the MVA–GFP constructs, or with corresponding amounts of PBS as a control (Fig. 3). Mice were sacrificed at day 8 or 28 and spleens were processed by ELISPOT assay to detect IFN-γ-secreting CD8+ T-cells. MVA-immunized mice induced significantly higher numbers of cells producing IFN-γ following GFP-specific peptide stimulation than mock-inoculated control mice (Fig. 3a). Interestingly, after the second immunization at day 21, MVA-myr–GFP induced significantly higher levels of GFP-specific CD8+ T-cells than either MVA–GFP or MVA-nls–GFP (Fig. 3b). Notably, the levels of GFP-specific CD8+ T-cells induced by infection with MVA-myr–GFP were similar to those seen after MVA-P7.5–GFP immunization. In addition, we monitored for MVA-specific CD8+ T-cells and confirmed very comparable F26–34 peptide-specific (Tscharke et al., 2006) responses for all MVA-based vaccines (Fig. 3c, d).

Our aim was to generate an MVA vector vaccine that optimizes the induction of antigen-specific T-cells. Recent data have increasingly highlighted the importance of antigen-specific T-cells for generating protective immunity against infectious diseases, particularly in the context of more complex viruses such as influenza virus and human immunodeficiency virus, which characteristically mutate their antigenic structure very rapidly (Brandler et al., 2010; Kremer et al., 2012a). Moreover, long-lived memory CD8+ T-cell immunity is considered important for cross-protection to ensure broader efficacy against different virus strains (Brown & Kelso, 2009). Such vaccines are urgently needed to control pathogens with pandemic potential such as influenza (Ahlers & Belyakov, 2010).

Previous studies have proposed cross-priming as the most important mechanism for antigen presentation upon primary immunization to induce efficient T-cell response (Gasteiger et al., 2007). Here, antigen-presenting cells such as dendritic cells package the antigen expressed by donor cells using an MHC class I molecule on their cell surface.

We analysed whether localizing the antigen to different cellular sites could affect the T-cell immunogenicity of recombinant MVA. For this purpose, we constructed recombinant MVA expressing GFP or GFP linked to either a nuclear localization signal or a cell membrane-locating myristoyl group. Unmodified GFP delivered by MVA was distributed equally throughout the infected cell and was not secreted, providing a good baseline to analyse the influence of selected cell compartments. Our immunostaining results confirmed the accumulation of GFP in different subcellular localizations. Indeed, for this purpose GFP may be superior to other commonly used model proteins, such as ovalbumin, which is glycosylated in the endoplasmic reticulum/Golgi compartments and secreted from the cell (Becker et al., 2014; Nörder et al., 2010).

The choice of the early Pvgf promoter should not only drive early protein synthesis but also avoid the potential hiding of antigens in viral factories formed at late times of infection (Katsafanas & Moss, 2007); both parameters should facilitate efficient endogenous antigen presentation, which is needed to appropriately compare the use of different localization signals. Moreover, recent in vitro studies demonstrated the unique transcription strength of Pvgf (Yang et al., 2015), which additionally recommends the use of this promoter.

The localization signals of the recombinant MVA–GFP viruses did not influence either their growth kinetics compared with non-recombinant WT virus or the expression levels of the modified antigens. Direct comparison of strict early expression induced by AraC treatment clearly indicated the much more efficient early gene expression by the Pvgf promoter compared with the well-established P7.5 promoter, as shown by GFP amounts detected in Western blot analysis. In vivo, we observed comparable activation levels of GFP epitope-specific CD8+ T-cells for all vaccines after single immunization. However, after boost vaccination, expression of myristoylated GFP significantly enhanced the induction

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of GFP CD8<sup>+</sup> T-cells compared with the nuclear localization signal or unmodified GFP. These data are relevant because they confirm that GFP accumulation on cellular membranes has beneficial effects for activating antigen-specific CD8<sup>+</sup> T-cells. Moreover, these results support the hypothesis of Gasteiger et al. (2007) suggesting that subcellular localization of target antigens could optimize the antigen characteristics to the requirements of the MVA vector system. Optimal interaction between the target antigen and the host cell system could then also result in enhanced cross-presentation and thereby induce an elevated immune response. Interestingly, early Pvgf in combination with myristoylation produced an immunization efficacy comparable to early/late P7.5–GFP. Pvgf is clearly the stronger early promoter, whereas the P7.5 promoter can be assumed to allow for higher levels of recombinant gene expression.
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when combining early and late transcriptional activities (Yang et al., 2015). However, based on previous work (Kastenmuller et al., 2007), we also expected a possible disadvantage of the early/late promoter P7.5 in boosting the CD8⁺ T-cell response, which we did not observe. Thus, it could be that the late gene expression provided by P7.5 does contribute to the in vivo amplification of GFP-specific CD8⁺ T-cells. Subsequent studies will be necessary to investigate precisely the effect of myristoylation on antigen cross-presentation and the activation of cytotoxic T-cells in the context of MVA early/late gene expression. In addition, while myristoylation can be expected to enhance the immunogenicity of MVA-produced antigens similar to GFP, it will be interesting to further test other target proteins including glycosylated and membrane-anchored antigens.

Taken together, our data support the idea that myristoylation could be a promising strategy for modifying antigens in the development of MVA-based vaccines against threatening infectious diseases. The recombinant MVA-myrt-GFP prototype that we developed here merits further analysis in the context of real antigens, for example the nucleoprotein antigen of influenza A virus.

**Acknowledgements**

We thank Sylvia Jany for excellent support in ELISPOT analysis and Ursula Klostermeier for expert help in animal work. This work was supported by European Union grant FLUNIVAC (602604).

**References**


