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ORIGINAL ARTICLE

Opposite effects of *Vaccinia* and modified *Vaccinia* Ankara on trained immunity

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Abstract

Vaccines such as *Vaccinia* or BCG have non-specific effects conferring protection against other diseases than their target infection, which are likely partly mediated through induction of innate immune memory (*trained immunity*). MVA85A, a recombinant strain of modified *Vaccinia* Ankara (MVA), has been suggested as an alternative vaccine against tuberculosis, but its capacity to induce positive or negative non-specific immune effects has not been studied. This study assesses whether *Vaccinia* and MVA are able to induce trained innate immunity in monocytes. Human primary monocytes were primed in an in vitro model with *Vaccinia* or MVA for 1 day, after which the stimulus was washed off and the cells were rechallenged with unrelated microbial ligands after 1 week. Heterologous cytokine responses were assessed and the capacity of MVA to induce epigenetic changes at the level of cytokine genes was investigated using chromatin immunoprecipitation and pharmacological inhibitors. Monocytes trained with *Vaccinia* showed significantly increased IL-6 and TNF-α production to stimulation with non-related stimuli, compared to non-trained monocytes. In contrast, monocytes primed with MVA showed significant decreased heterologous IL-6 and TNF-α responses, an effect which was abrogated by the addition of a histone methyltransferase inhibitor. No effects on H3K4me3 were observed after priming with MVA. It can be thus concluded that *Vaccinia* induces trained immunity in vitro, whereas MVA induces innate immune tolerance. This suggests the induction of trained immunity as an immunological mechanism involved in the non-specific effects of *Vaccinia* vaccination and points to a possible explanation for the lack of effect of MVA85A against tuberculosis.

Keywords Trained immunity · *Vaccinia* · Modified *Vaccinia* Ankara · Heterologous effects

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Abbreviations

BCG	Bacillus Calmette-Guérin
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MTA	5′-Deoxy-5′methylthioadenosine
MVA	Modified *Vaccinia* Ankara
OPV	Oral polio vaccine
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
VACV	*Vaccinia* virus

Introduction

Vaccinia virus (VACV) belongs to the family of *Poxviridiae* and has been used to vaccinate against smallpox, the severe disease caused by the related *Variola* virus. Vaccination

campaigns with *Vaccinia* were very successful, ultimately leading to the eradication of smallpox in 1977, which prompted the World Health Organization to recommend stopping *Vaccinia* vaccination in 1980.

In addition to their specific protective effects against their target diseases, vaccines may have non-specific effects, i.e., effects on morbidity and mortality which cannot be explained by the prevention of the target-disease [1]. Several live vaccines, such as BCG, measles vaccine, and oral polio vaccine (OPV), have been shown in randomized clinical trials to decrease overall mortality more than can be explained by prevention of tuberculosis, measles, or polio, respectively [2–6]. Likewise, several observational studies conducted after the eradication of smallpox indicate that vaccinia may decrease overall mortality among adults in Guinea-Bissau and Denmark [7–9], and *Vaccinia* vaccination has been linked to protection against non-Hodgkin lymphoma and melanoma [10, 11]. Whereas the mechanisms behind the non-specific protective effect of *Vaccinia* have not yet been investigated, the non-specific protection elicited by BCG is explained, at least in part, by the induction of trained immunity, a term used to describe epigenetically mediated memory properties of the innate immune system [12, 13].

Modified *Vaccinia* Ankara (MVA) was developed in the late 1950s as a highly attenuated strain of VACV. Extended serial passage of the chorioallantois VACV Ankara (CVA) resulted in a host range-restricted virus, which is replication-deficient in most mammalian cells [14]. This strain is highly attenuated and has been shown to have a more favorable safety profile compared to VACV [15]. MVA85A is a recombinant strain of MVA expressing the immunodominant *Mycobacterium tuberculosis* protein 85A and is being studied as a heterologous booster vaccination for BCG vaccination [16]. Although MVA85A boosting of BCG vaccinated infants and adults resulted in induction of antigen-specific Th1 and Th17 cells, no effect on protection against tuberculosis has been observed in two trials [16, 17]. Given the important non-specific effects of BCG vaccination, it is necessary that new tuberculosis vaccine candidates be assessed for their capacity to elicit non-specific positive or negative immunological effects. The present study aims to investigate whether *Vaccinia* and MVA can induce trained immunity in an in vitro model.

Materials and methods

PBMC isolation

PBMCs were isolated from buffy coats from healthy volunteers (Sanquin, Nijmegen, The Netherlands, and Rigshospitalet, Copenhagen, Denmark). For the experiments performed with *Vaccinia*, a total of 8 donors were used (1 male, 5 female, 2 unknown), and for experiments performed with MVA, 11 donors were used (1 male, 2 females, 8 unknown). Briefly, blood was diluted in pyrogen-free saline and PBMCs were separated over Ficoll-Pacque (GE Healthcare, UK). Cells were washed three times in cold PBS and Percoll isolation of monocytes was performed as described previously [18]. After isolation, cells were resuspended in RPMI-1640 supplemented with gentamycin (10 μg/ml), pyruvate (10 mM), and glutamax (10 mM). Monocytes were counted using a Coulter counter and adjusted to 1×10^6/ml. A total of 1×10^5 monocytes were added to polystyrene 96 wells flat bottom plates (Corning, NY, USA), and cells were left to adhere for 1 h, after which they were washed once with warm PBS.

In vitro trained immunity model

Adherent monocytes were primed by stimulation with RPMI (negative control), MVA (Imvamune; Bavarian-Nordic; Denmark), *Vaccinia*, or β-1,3-D-glucan (5 μg/ml; kindly provided by professor David Williams (TN, USA)). The *Vaccinia* used in this study was a single batch of the smallpox vaccine (Copenhagen VACV strain, produced in April 1976 at Statens Serum Institut (Copenhagen, Denmark)) as harvested exudate from wounded skins of inoculated calves. The vaccine has been bacteriologically tested and stored at − 80 °C until use.

After 24 h of stimulation, cells were washed once using warm PBS and RPMI supplemented with 10% human pool serum was added. On day 3, culture medium was refreshed. On day 6, culture medium was removed and cells were restimulated using *Escherichia coli* 005:B5 lipopolysaccharide LPS (10 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), Pam3Cys (10 μg/ml; EMC Microcollections, Tuebingen, Germany), or culture medium alone (negative control). After 24 h of restimulation, cell culture supernatants were harvested and stored at − 20 °C until analysis.

For selected experiments of training with MVA, the histone methyltransferase inhibitor 5′-deoxy-5′ methylthioadenosine (MTA, 1 mM; Sigma-Aldrich) was added for the first 24 h of the experiment. (See Fig. 1 for a schematic overview of the experimental setup.)

Chromatin immunoprecipitation

In order to assess epigenetic markers associated with inflammatory changes, monocytes (10×10^6 cells in 2 ml volume) were added to six-well plates and left to adhere for 1 h, after which they were washed with PBS. Adherent monocytes were stimulated with RPMI or MVA (1×10^6 tissue culture infectious dose $(TCID)_{50}$/ml) for 24 h after which the stimuli were washed off. Cells were left to rest and at day 6 were harvested and fixated in methanol-free formaldehyde (1%). After fixation, cells were sonicated and immunoprecipitation was

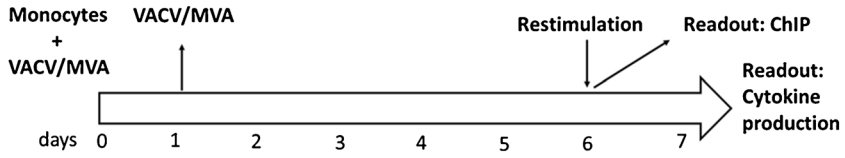

Fig. 1 Schematic overview of in vitro trained immunity model. Monocytes were primed for 24 h using VACV (1:1000 and 1:10.000 dilution), MVA (1×10^4–1×10^5–1×10^6 $TCID_{50}$/ml), β-glucan (5 μg/ml), or culture medium alone. After washing away, the priming stimulus cells were rested for 5 days, after which the cells were restimulated with RPMI, LPS (10 ng/ml), or Pam3Cys (10 μg/ml). For selected experiments, monocytes were harvested on day 6 for further processing for chromatin immunoprecipitation

performed using antibodies against H3K4me3 (Diagenode, Seraing, Belgium), and DNA was further processed for qPCR analysis. Samples were analyzed by comparative threshold method in which myoglobin was used as a negative control and H2B as a positive control, according to the manufacturer's instructions. The following primers were used for this analysis: myoglobin forward AGCATGGTGCCACT GTGCT; myoglobin reverse GGCTTAATCTCTGC CTCATGAT; H2B forward TGTACTTGGTGACG GCCTTA; TNF forward GTGCTTGTTCCTCAGCCTCT; TNF reverse ATCACTCCAAAGTGCAGCAG; IL-6 forward AGGGAGAGCCAGAACACAGA; IL-6 reverse GAGTTTCCTCTGACTCCATCG.

Cytokine measurements

IL-6 and TNF-α in the supernatants of stimulated monocytes were measured using commercially available ELISA kits from Sanquin (Amsterdam, The Netherlands; IL-6) and R&D Systems (Minneapolis, MN, USA; TNF-α).

Statistical analysis

Data was analyzed by use of the Wilcoxon signed-rank test to compare raw cytokine levels of cells trained with VACV or MVA to RPMI control. All calculations were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A two-sided p value of $p < 0.05$ was considered statistically significant. All data are shown as means ± SEM (^p = 0.06; *$p < 0.05$; ** = $p < 0.01$).

Results

Characterization of *Vaccinia* activity

Since the capacity of the smallpox vaccine to stimulate cytokine production in monocytes in the in vitro system was unknown, we initially performed a dose-response stimulation experiment, in which human monocytes from two donors were cultured for 24 h in a tenfold dilution series of *Vaccinia* in RPMI, starting at 1:10. The cells were inspected by light microscope on days 1, 4, and 5, and lactate dehydrogenase (LDH) release (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega) was measured in supernatants on days 1, 4, and 5 for 1:10 and 1:1000 dilutions to assess cell toxicity. *Vaccinia* induced rapid cell death in dilutions of 1:10 and 1:100 with practically no cells remaining after 1 day, whereas dilution 1:1000, but not higher dilutions, seemed to only slightly reduce the number of cells. Dilutions 1:1000 and 1:10,000 induced clearly observable morphological changes on day 4, when they appeared as larger, darker, and more round-shaped cells. Cells that were primed with *Vaccinia* in a 1:100,000 dilution did not show microscopic phenotypical changes. On day 1, LDH release was highly prominent in the 1:10 dilution, but only slightly increased in the 1:1000 dilution relative to medium alone; LDH on days 4 and 5 was not different to the background levels (data not shown). Based on this dose-response experiment, we decided to use VACV in 1:10,000 and 1:1000 dilutions to assess the capacity of *Vaccinia* to induce trained immunity. Due to insufficient materials being available, we unfortunately did not have the possibility to determine viral titers, and we were unable to find information about the batch of *Vaccinia* which could be used as a proxy to viral titers.

Vaccinia induces increased pro-inflammatory responses in an in vitro trained immunity model

To assess the capacity of *Vaccinia* to induce trained immunity, adherent monocytes were primed for 24 h with *Vaccinia*, β-glucan, or culture medium. After 24 h, the priming stimuli were washed off and monocytes were left to rest for 1 week before being restimulated with culture medium alone, the TLR4 ligand LPS, or the TLR2 ligand Pam3Cys, respectively. Restimulation with culture medium alone resulted in similar, very low cytokine production in all training conditions (data not shown). IL-6 and TNF-α production after stimulation with LPS and Pam3Cys was significantly upregulated in cells that were primed with *Vaccinia* compared to cells exposed to culture medium alone (Fig. 2). Remarkably, enhancement of TNF-α responses upon restimulation was more pronounced in *Vaccinia*-primed cells compared to β-glucan, a prototypical inducer of trained immunity [19]. Light microscopy did not indicate changes in cellular survival between the different conditions during the experimental period.

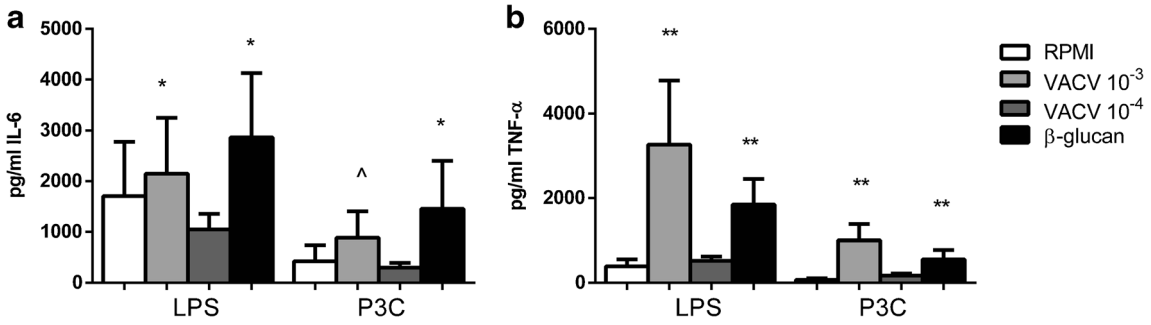

Fig. 2 VACV priming induces increased pro-inflammatory cytokine production after 1 week. **a** IL-6 production after restimulation with LPS or Pam3Cys is increased when cells are trained with β-glucan and VACV. **b** TNF-α production after restimulation with LPS or Pam3Cys is increased when cells are trained with β-glucan or VACV. (Data shown as mean ± SEM. The Wilcoxon signed-rank test compared to RPMI training. $N = 8$; $^p = 0.06$, $*p < 0.05$, $**p < 0.01$)

MVA inhibits pro-inflammatory responses in an in vitro trained immunity model

To investigate whether MVA induces trained immunity, experiments similar to the above were performed using MVA. Cytokine production in conditions primed with culture medium alone was all below detection limit of the ELISA kits. When monocytes were primed with MVA 1 week before restimulation, IL-6 and TNF-α production to restimulation with LPS and Pam3Cys was significantly reduced compared to monocytes primed with culture medium alone (Fig. 3). Interestingly, this effect seemed to be dose-dependent, with a high dose of MVA (1×10^6 $TCID_{50}$/ml) leading to decreased cytokine responses, whereas lower doses (1×10^4 and 1×10^5 $TCID_{50}$/ml) did not modulate heterologous cytokine responses after 1 week.

To exclude induction of cell toxicity and cell death in MVA-primed cells, LDH was measured in cell supernatants 24 h, 3 days, and 6 days after priming with MVA. LDH measurements between MVA- and RPMI-primed cells did not differ, suggesting that the decrease in cytokine production after MVA priming is not due to increased cell death (data not shown).

Inhibition of histone methyltransferase activity abrogate MVA induced tolerance

Since we previously found that the induction of trained immunity by β-glucan and BCG is epigenetically mediated, we assessed the role of epigenetic modulation in the induction of tolerance after MVA priming. When the histone methyltransferase inhibitor MTA was added during the priming stimulation with MVA, downregulation of pro-inflammatory cytokine responses upon restimulation after 1 week was abrogated (Fig. 4). This suggests that the inhibitory effect of MVA on heterologous responses takes place via an epigenetically mediated mechanism involving methylation of histones.

MVA does not decrease H3K4me3 of pro-inflammatory cytokines

Trained immunity elicited by β-glucan or BCG is associated with increases in the histone mark H3K4me3, which is associated with increased transcription. To assess the impact of MVA on H3K4me3, cells were detached at day 6 and fixated for chromatin immunoprecipitation. Cells that were primed with MVA showed a non-significant increase in H3K4me3 on the IL-6 promotor and no difference in H3K4me3 on the

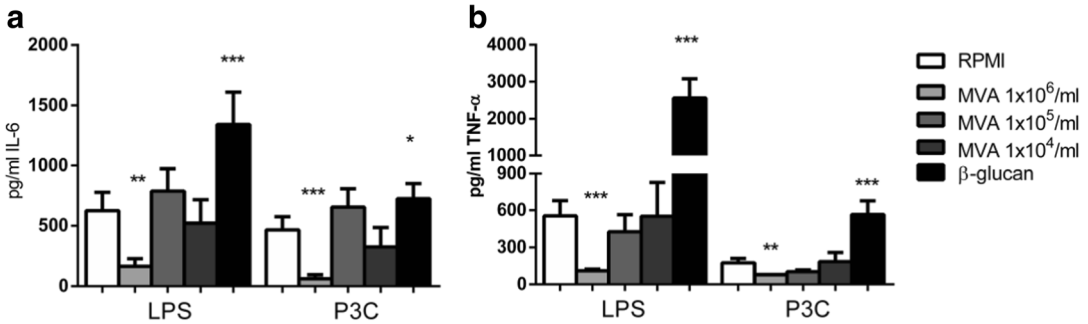

Fig. 3 MVA priming decreases pro-inflammatory cytokine production after 1 week. **a** IL-6 production after restimulation with LPS or Pam3Cys is increased when cells are trained with β-glucan, but decreased when cells are trained with MVA. **b** TNF-α production after restimulation with LPS or Pam3Cys is increased when cells are trained with β-glucan, but decreased when cells are trained with MVA. (Data shown as mean ± SEM. The Wilcoxon signed-rank test; $N = 11$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

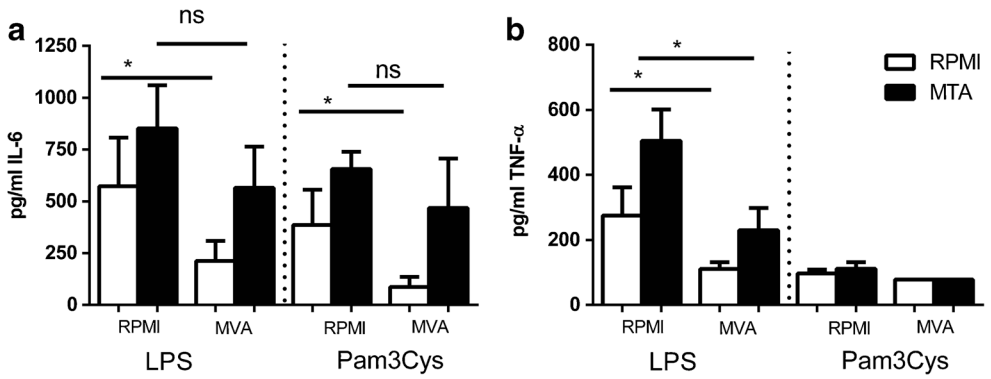

Fig. 4 Addition of MTA abrogate induction of innate immune tolerance by MVA. MTA (1 mM) was added during priming stimulation. **a** IL-6 production after restimulation with LPS or Pam3Cys is decreased when cells are trained with MVA. When MTA is added during training phase of the experiment, this decrease is abrogated. **b** TNF-α production after restimulation with LPS or Pam3Cys is decreased when cells are trained with MVA. Addition of MTA does not influence decreased TNF-α production induced by MVA. (Data shown as mean ± SEM. The Wilcoxon signed-rank test; $N = 7$, $^*p < 0.05$, ns not significant)

TNF-α promotor compared to cells primed with RPMI (Fig. 5).

Discussion

In this study, we show that *Vaccinia* and MVA have opposite effects on trained immunity in vitro. *Vaccinia* induced trained immunity with potentiation of pro-inflammatory cytokine responses to unrelated stimuli when cells were restimulated after 1 week of the initial exposure. In contrast, MVA induced a dose-dependent innate immune tolerance, with decreased pro-inflammatory cytokine responses to unrelated stimuli. Interestingly, this tolerizing effect of MVA was abrogated when a methyltransferase inhibitor was used during priming. This suggests that increases in histone methylation, likely of repressive marks since they lead to decreased cytokine production, are involved in this process.

Observational studies have shown that *Vaccinia* has non-specific effects which lead to decreased overall mortality [7, 9]. Furthermore, some studies have linked *Vaccinia* with protection against melanoma and non-Hodgkin lymphoma [10, 11]. This effect seems to be analogous to the non-specific effects of BCG vaccination. Recent studies have suggested that the non-specific effects of BCG are at least partly mediated through induction of trained immunity [13]. Our finding that *Vaccinia* induces trained immunity in vitro supports that the non-specific effects of *Vaccinia* might be partly mediated by innate immune training. Several other studies that have shown heterologous immunological effects of *Vaccinia* further support this assumption. Spleen cells from hamsters that were vaccinated with VACV showed increased cytotoxic activity against cells that were infected with herpes simplex virus (HSV), an effect which seemed to be mediated by NK cells [20]. Similarly, monocytes and macrophages from mice vaccinated with *Vaccinia* showed increased IL-6 and TNF-α responses to HSV-infected cells, and peritoneal cells from *Vaccinia* vaccinated mice show increased reactive oxygen species production to stimulation with zymosan or *Candida albicans* [21, 22]. In contrast, an in vitro study showed that VACV infection of dendritic cells led to decreased IL-6 and TNF-α response to

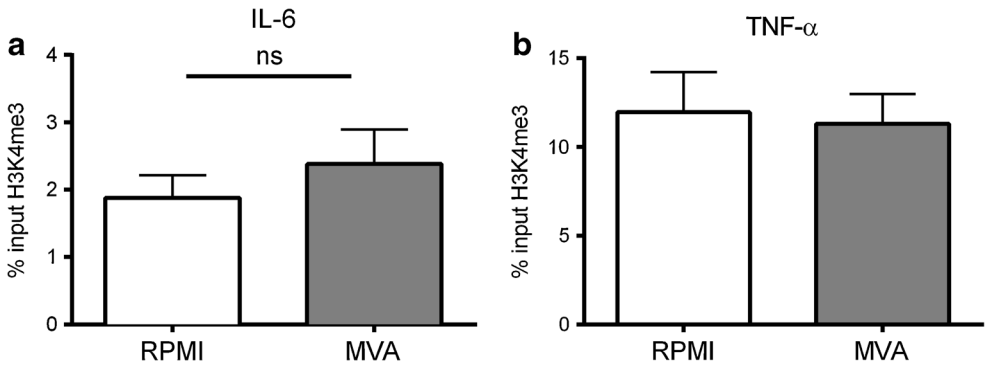

Fig. 5 Priming with MVA does not influence H3K4me3 on promotors of pro-inflammatory cytokine genes. **a** % input of H3K4me3 on promotor region of IL-6 of monocytes primed with MVA (1×10^6 $TCID_{50}$/ml) 6 days earlier. **b** % input of H3K4me3 on promotor region of TNF-α of monocytes primed with MVA (1×10^6 $TCID_{50}$/ml) 6 days earlier. (Data shown as mean ± SEM. The Wilcoxon signed-rank test; $N = 8$, ns not significant)

LPS stimulation [23]. However, this study used dendritic cells and restimulated VACV-infected cells 2 h after infection, whereas we have looked at the effects in adherent monocytes after 1 week, which may explain this difference since it has been shown that there are differences in innate cytokine responses between these two cell types [24]. Recent data show that at least several days of rest are needed for trained immunity to be established [25]. Results from an in vivo vaccination study comparing gene expression before and after *Vaccinia* vaccination further supports that *Vaccinia* can induce trained immunity since it showed that up to 55 days after vaccination, several innate immune genes were still upregulated, including IL-8, CXCR2, and IL-18 [26]. Interestingly, several studies point to a role of TLR2 in innate immune recognition of VACV, and engagement of TLR2 signaling in hematopoietic stem cells has been shown to lead to functionally altered progenitor cells [27, 28]. Therefore, one could speculate that TLR2-mediated signaling by *Vaccinia* leads to the observed long-term, non-specific epidemiological effects of *Vaccinia*.

Our finding that MVA induces innate immune tolerance is of importance in the light of the clinical trials using MVA as a new smallpox vaccine candidate and trials investigating MVA as a vector for vaccination against other diseases. This might be most important for MVA85A, a recombinant form of MVA expressing the immunodominant *M. tuberculosis* protein 85A, which is extensively being studied as a heterologous booster vaccine for BCG. Although MVA85A induces specific, adaptive immunity against *M. tuberculosis*, the vaccine did not lead to increased protection against tuberculosis infection [16, 17]. The fact that MVA did not induce trained immunity in our model, but on the contrary induced tolerance, might suggest that the lack of effect of MVA85A on tuberculosis could be caused by its inability to train innate immune responses to *M. tuberculosis*. In this light, it is interesting to note that there are data to suggest that early clearance of *M. tuberculosis* by the innate immune system might be of importance to prevent tuberculosis infection [29, 30]. Thus, our results indicate that new vaccines against tuberculosis and other recombinant vaccines using MVA as a delivery virus should also be assessed for their capacity to induce trained immunity and non-specific effects.

The fact that priming with *Vaccinia* and MVA leads to different phenotypes in our study suggests that there are important differences in how these two vaccines are recognized and how they activate innate immune cells. Indeed, differences have been noted between VACV and MVA in terms of innate immune activation, with *Vaccinia* virus Western Reserve strain and *Vaccinia* virus Copenhagen strain failing to induce NF-κB signaling, but infection with MVA showing induction of NF-κB signaling [31]. In this light, it is interesting to note that induction of NF-κB is associated with LPS tolerance, in which IL-6 and TNF-α responses are also downregulated [32–34].

Several limitations to our study should be noted. First, all experiments were performed in vitro and may not directly translate to the in vivo effects of these vaccines. Second, the *Vaccinia* we used in this study was obtained from an old stock, with unknown concentration and strength. A serial dilution was tested for activity in terms of microscopic phenotypical changes and cell death measured by LDH release, but the actual concentration used is unknown. Third, the vaccination production procedure (obtained from exudate of inoculated cows) might make impurities possible, including antigens which may also have immune stimulating potential. This makes it difficult to make a direct comparison between MVA and *Vaccinia* and to extrapolate the results to the in vivo results of *Vaccinia*.

In conclusion, our study shows that *Vaccinia* and MVA show opposite effects in an in vitro model of trained immunity, with *Vaccinia* inducing innate immune training leading to increased heterologous cytokine responses upon restimulation and MVA leading to a tolerant phenotype with decreased heterologous cytokine responses upon restimulation. These results support the notion that *Vaccinia* has non-specific effects and highlights the importance of investigating new substitute tuberculosis vaccines for their non-specific immunological effects and induction of trained immunity.

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Author contributions CSB, MGN, RvC, PA, and BAB conceived the study. BAB and KJJ performed the in vitro experiments and analyzed the data. MGN and AF supervised the in vitro experiments. BAB wrote the first draft of the article. All authors contributed to and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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