

The Canarypox Virus Vector ALVAC Induces Distinct Cytokine Responses Compared to the Vaccinia Virus-Based Vectors MVA and NYVAC in Rhesus Monkeys

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Despite the growing use of poxvirus vectors as vaccine candidates for multiple pathogens and cancers, their innate stimulatory properties remain poorly characterized. Here we show that the canarypox virus-based vector ALVAC induced distinct systemic proinflammatory and antiviral cytokine and chemokine levels following the vaccination of rhesus monkeys compared to the vaccinia virus-based vectors MVA and NYVAC. These data suggest that there are substantial biological differences among leading poxvirus vaccine vectors that may influence resultant adaptive immune responses following vaccination.

Poxvirus vectors are used for vaccination against multiple pathogens, including human immunodeficiency virus type 1 (HIV-1) and cancer (1–3). A recent study that used the canarypox virus-based vector ALVAC with a HIV-1 gp120 protein boost showed 31.2% efficacy against HIV-1 infection in a phase III trial in Thailand (RV144) (4). While analysis of data from the RV144 trial has indicated several possible correlates of vaccine-induced protection (5), the innate immune stimulatory properties of ALVAC vectors and how they might influence adaptive immune responses remain poorly described. In this study, we compared innate immune stimulation as measured by serum cytokine and chemokine levels following vaccination of rhesus monkeys with ALVAC and two vaccinia virus-based vectors, modified vaccinia virus Ankara (MVA) (6) and New York vaccinia virus (NYVAC) (7).

We initiated studies by assessing serum cytokine and chemokine levels following vaccination with the replication-incompetent ALVAC, MVA, or NYVAC vector in 32 rhesus monkeys (*Macaca mulatta*). All studies with animals and human cells were approved by the relevant Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB). Monkeys ($n = 8$ to 12/group) were injected intramuscularly (i.m.) with 1×10^8 PFU of the ALVAC, MVA, or NYVAC vector expressing simian immunodeficiency virus (SIV) Gag, Pol, and Env (8–10). All of the vectors used in these studies were similar in purity and infectivity. Sera were collected on days 0, 1, 3, 7, and 14 following vaccination. Systemic cytokine and chemokine levels were assessed by Luminex assays and enzyme-linked immunosorbent assays (ELISAs) as previously described (11). Longitudinal analysis of systemic cytokine and chemokine levels following ALVAC vaccination revealed potent but transient induction of proinflammatory cytokines and chemokines on day 1 postimmunization (Fig. 1A and B). In contrast, lower induction of proinflammatory cytokines and chemokines was observed in monkeys vaccinated with MVA or NYVAC (Fig. 1A and B). Animals that received ALVAC displayed greater fold induction over the averaged group baseline than those that received MVA or NYVAC for interleukin-1 β (IL-1 β) (411- and 408-fold greater induction, respectively; $P = 0.0002$ and $P < 0.0001$, respectively; Mann-Whitney U Test), IL-6 (83- and 6.3-fold greater induction, respectively; $P = 0.0003$ and $P =$

0.0043, respectively), tumor necrosis factor alpha (TNF- α) (48- and 29-fold greater induction, respectively; $P = 0.0002$ and $P < 0.0001$, respectively), monocyte chemoattractant protein 1 (MCP-1) (4- and 3-fold greater induction, respectively; $P = 0.0002$ and $P < 0.0001$, respectively), macrophage inflammatory protein alpha (MIP-1 α) (37- and 79-fold greater induction, respectively; $P = 0.003$ and $P = 0.0004$, respectively), and MIP-1 β (9- and 10-fold greater induction, respectively; $P = 0.0002$ and $P < 0.0001$) at day 1 following vaccination. These data show that ALVAC induced a distinct proinflammatory response compared to MVA and NYVAC in rhesus monkeys.

Unsupervised hierarchical clustering of animals based upon fold induction at day 1 postvaccination over the individual baselines of all of the cytokines and chemokines analyzed indicated further that the responses elicited by ALVAC, MVA, and NYVAC are all distinct (Fig. 1C). ALVAC induced a distinct proinflammatory cytokine milieu, but differences were also observed between MVA and NYVAC for several cytokines, such as IL-6, transforming growth factor alpha, and IL-1RA (Fig. 1C). Together, these data indicate that ALVAC, MVA, and NYVAC all induce qualitatively distinct cytokine profiles following vaccination.

We next analyzed the impact of priming with a heterologous vector on elicited cytokines following boosting with ALVAC, MVA, or NYVAC. Rhesus monkeys ($n = 12$ /group) were injected i.m. with 3×10^{10} viral particles of an adenovirus serotype 26 (Ad26) vector expressing SIV Gag, Pol, and Env. Twenty-four weeks following priming vaccination, animals were boosted i.m. with 1×10^8 PFU of ALVAC, MVA, or NYVAC expressing SIV Gag, Pol, and Env. Longitudinal analysis of serum cytokines and chemokines following the boost immunization with ALVAC, MVA, or NYVAC revealed clear induction of proinflammatory

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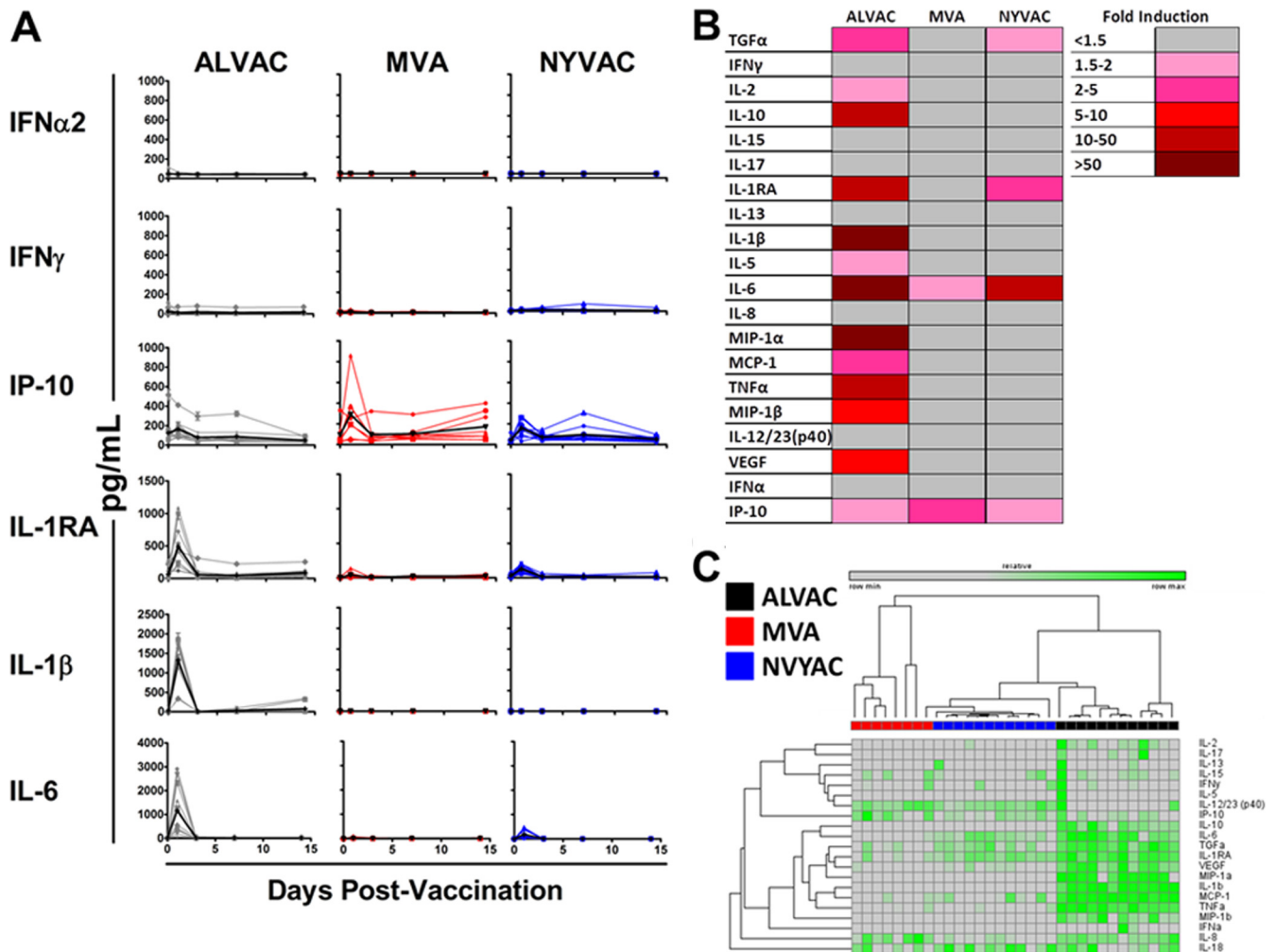


FIG 1 Serum cytokine and chemokine concentrations of rhesus monkeys following poxvirus vector vaccination. Rhesus monkeys ($n = 8$ to 12 /group) were inoculated i.m. with 1×10^8 PFU of ALVAC, MVA, or NYVAC expressing SIV Gag, Pol, and Env. Sera were collected on days 0, 1, 3, 7, and 14 following vaccination, and systemic levels of cytokines and chemokines were analyzed by Luminex assays and ELISAs. (A) Systemic levels of selected cytokines and chemokines following vaccination with the various poxvirus vectors, with individual levels shown as colored lines and group means over time shown as black lines. (B) Mean fold induction of all cytokines and chemokines at day 1 postvaccination of rhesus monkeys with poxvirus vectors. The values shown are group mean fold induction over the averaged group baseline. (C) Fold cytokine induction over the grouped baseline of rhesus monkeys following vaccination with ALVAC, MVA, and NYVAC. Monkeys are clustered in an unsupervised hierarchical analysis based on individual fold induction over the baseline values of all of the cytokines. Clustering analysis was performed in Gene-E. Values are log individual fold induction of the indicated cytokines and chemokines over the grouped baseline at day 1 following vaccination with the various poxvirus vectors.

and antiviral cytokines such as IL-1 β , IL-6, gamma interferon (IFN- γ), and IFN- γ -induced protein 10 (IP-10), on day 1 following the boost (Fig. 2A). However, the cytokine levels elicited following boosting were lower than those observed following priming for all of the vectors, and particularly for ALVAC (Fig. 1A and 2A). Analysis of the average fold induction over the group average baseline indicated that boosting with ALVAC, MVA, or NYVAC induced comparable cytokine profiles, characterized by IL-1 β , IL-6, IL-1RA, IFN- γ , and IP-10 (Fig. 2B). These data suggest that Ad26 priming attenuated innate cytokine induction as measured by serum cytokine profiles of ALVAC-, MVA-, or NYVAC-boosted animals. We suspect that this may be related to enhanced vector clearance as a result of the adaptive immune responses elicited by the priming immunization, although other factors may play a role.

We next sought to assess cytokine and chemokine induction by ALVAC, MVA, and NYVAC *in vitro* in freshly isolated human peripheral blood mononuclear cells (PBMC). Fresh human PBMC ($n = 4$ /group) were isolated by Ficoll-Hypaque density gradient centrifugation (12). In order to include all of the possible cell types shown to be infectible with poxvirus vectors, whole PBMC were infected with the ALVAC, MVA, or NYVAC vector at a multiplicity of infection (MOI) of 10 PFU/cell (13). Culture supernatant was analyzed for cytokine and chemokine levels 24 h postinfection by Luminex assays. Similar to our *in vivo* results, stimulation of human PBMC with ALVAC resulted in higher levels of proinflammatory and IFN-related antiviral cytokines and chemokines than did stimulation with MVA or NYVAC (Fig. 3A). Stimulation of human PBMC with ALVAC elicited higher levels than that with MVA or NYVAC for IFN- γ (41- and 334-fold

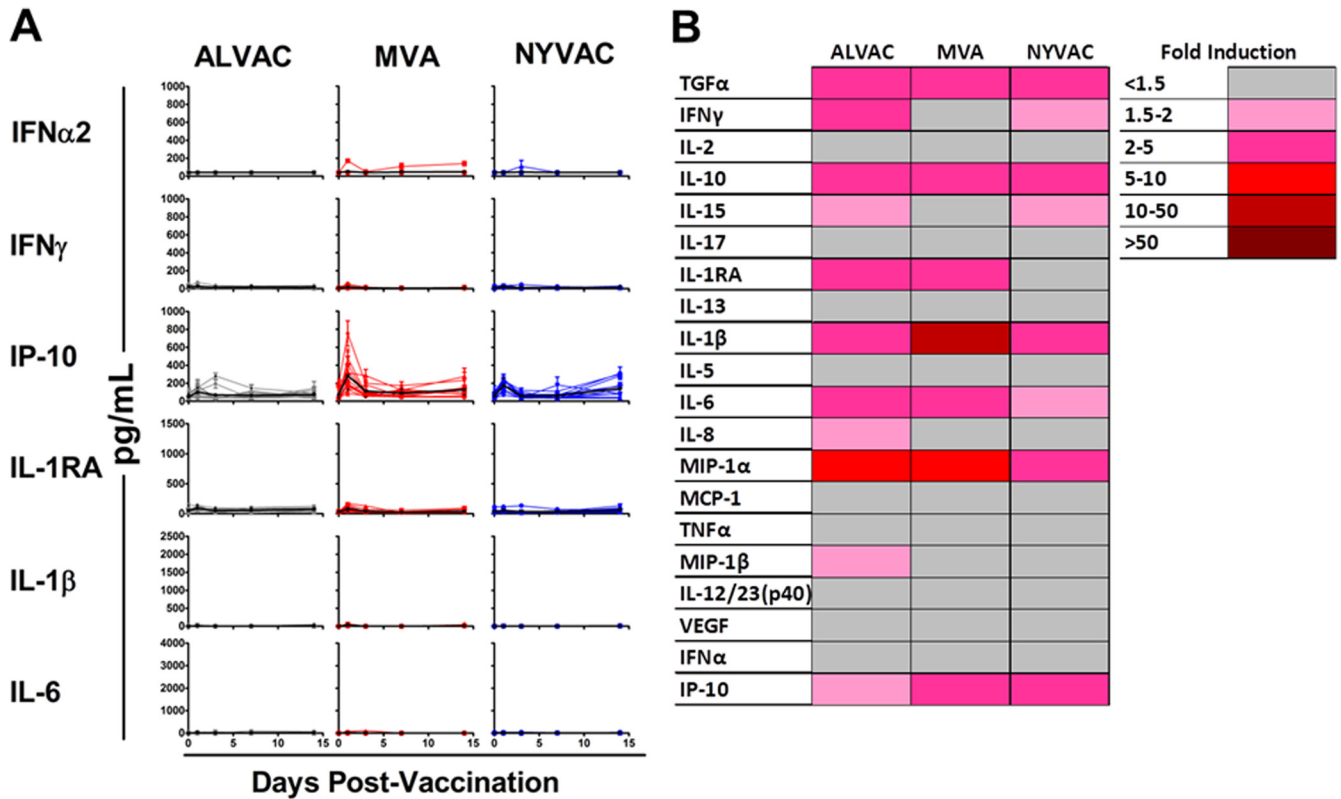


FIG 2 Serum cytokine and chemokine concentrations of rhesus monkeys following poxvirus vector boosting. Rhesus monkeys ($n = 12/\text{group}$) were primed i.m. with 3×10^{10} viral particles of Ad26 expressing SIV Gag, Pol, and Env and then boosted i.m. with 1×10^8 PFU of the ALVAC, MVA, or NYVAC vector expressing transgenes. Sera were collected on days 0, 1, 3, 7, and 14 following vaccination, and systemic levels of cytokines and chemokines were analyzed by Luminex assays and ELISAs. (A) Systemic levels of selected cytokines and chemokines following boosting with the various poxvirus vectors, with individual levels shown as colored lines and group means over time shown as black lines. (B) Mean fold induction of all cytokines and chemokines in rhesus monkeys at day 1 postboosting with poxvirus vectors. The values shown are group mean fold induction over the averaged group baseline prior to boosting with the various poxvirus vectors.

greater levels, respectively; both $P = 0.0286$, Mann-Whitney U test), IL-1β (37- and 315-fold greater levels, respectively; both $P = 0.0286$), IL-6 (3- and 15-fold greater levels, respectively; $P = 0.0571$ and $P = 0.0286$, respectively), and TNF-α (7- and 17-fold greater levels, respectively; both $P = 0.0286$). These *in vitro* results are comparable to our *in vivo* findings, with a few notable exceptions, such as a lack of difference observed in the elicitation of IFN-α2 *in vitro*.

We sought to assess the contributions of various PBMC subsets, some of which were previously shown to be readily transduced by vaccinia virus, to the production of cytokines and chemokines elicited by the ALVAC, MVA, and NYVAC vectors (13). Human PBMC ($n = 2$ to 6/group) were isolated as described above and depleted of T cells, monocytes and macrophages (MonoMac), myeloid dendritic cells, or plasmacytoid dendritic cells (pDC) by magnetic separation as previously described (11). Depletion was confirmed by flow cytometry (data not shown). Cells were then infected with ALVAC, MVA, or NYVAC at an MOI of 10 PFU/cell. Supernatants were analyzed 24 h postinfection for elicited cytokines and chemokines by Luminex analyses. Depletion of pDC led to a marked reduction in the levels of IFN-α2 (16-fold lower induction; $P = 0.0022$) elicited in response to ALVAC stimulation relative to those in unseparated cells (Fig. 4). Depletion of MonoMac resulted in reduced induction of IL-1β and TNF-α (117- and 37-fold lower induction; both $P = 0.0043$)

in response to ALVAC stimulation. Depletion of T cells markedly decreased the levels of elicited IFN-γ (15-fold lower induction; $P = 0.0087$) following ALVAC stimulation. A similar trend was observed with lowered levels of elicited IL-1β and TNF-α in response to MVA or NYVAC stimulation following depletion of MonoMac. Interestingly, pDC depletion had a more modest impact on IFN-α2 secretion following MVA or NYVAC stimulation than did ALVAC stimulation. Taken together, these results indicate that multiple PBMC subsets likely contribute to the overall cytokine milieu elicited by the ALVAC, MVA, and NYVAC vectors.

In this study, we show that the innate immune profiles elicited by ALVAC, MVA, and NYVAC are all distinct. ALVAC elicited an innate immune response qualitatively and quantitatively different from that elicited by MVA and NYVAC both *in vivo* in rhesus monkeys and *in vitro* in human PBMC, characterized by a higher induction of proinflammatory and IFN-related antiviral cytokines and chemokines on day 1 following immunization. Moreover, MVA and NYVAC also proved different, although these differences were less striking. The stimulatory phenotypes observed following priming with ALVAC, MVA, or NYVAC were all reduced when these poxvirus vectors were used as a boost. Furthermore, ALVAC's stimulatory phenotype was influenced by several PBMC subsets such as T cells, MonoMac, and pDC. These data suggest potentially important biological differences among these three

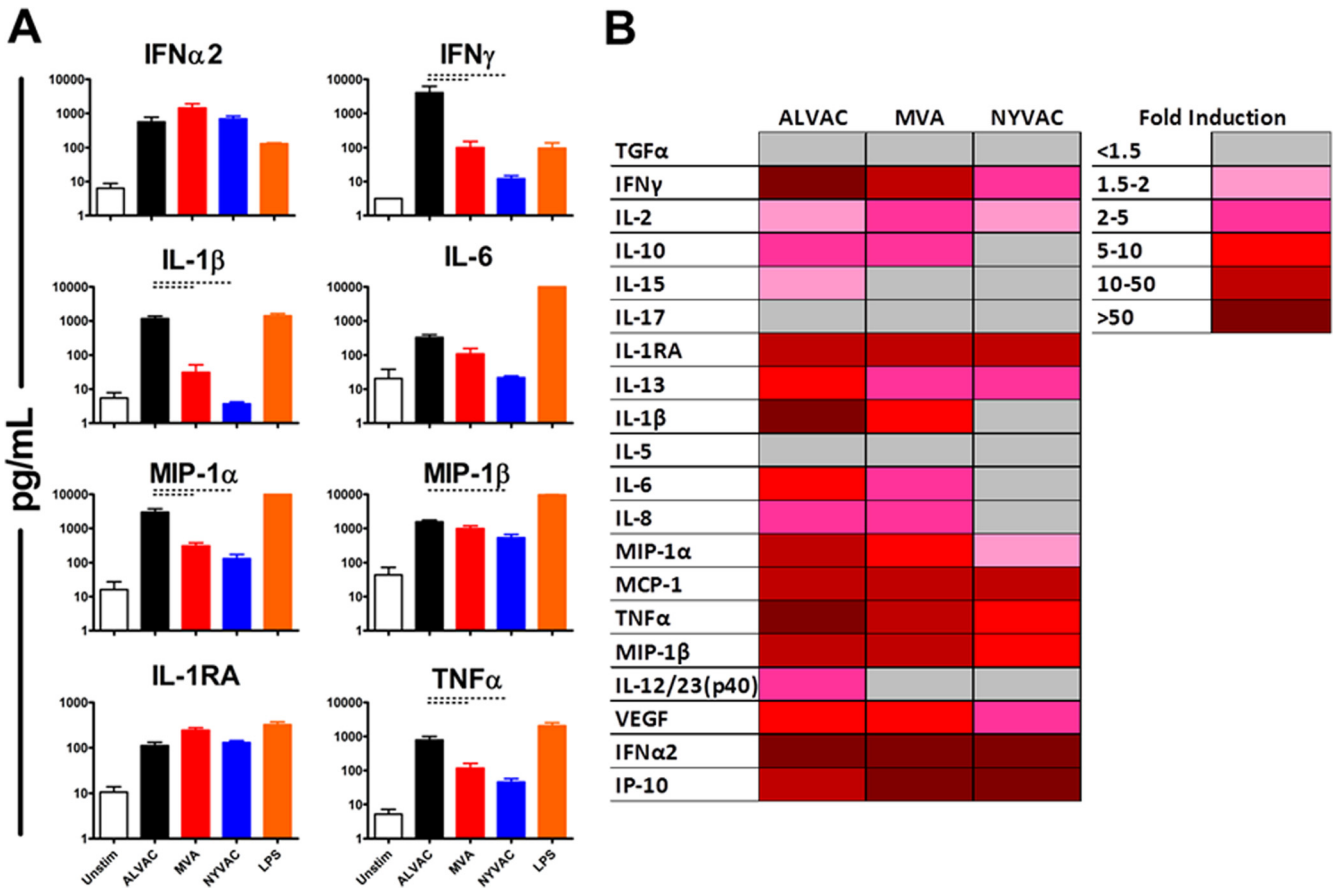


FIG 3 Induction of cytokines and chemokines by poxvirus vector stimulation of human PBMC. (A) Fresh whole human PBMC ($n = 4$ /group) were stimulated at an MOI of 10 PFU/cell with the ALVAC, MVA, or NYVAC vector expressing no transgene. Cytokine and chemokines were measured 24 h postinfection by Luminex assays. Lipopolysaccharide (LPS; 1 ng/ml) was included as the positive control. The data are means and standard errors of the means. Bars indicate P values of <0.05 by Kruskal-Wallis tests. (B) Mean fold induction of all cytokines and chemokines at 24 h postinfection of human PBMC with poxvirus vectors. The values shown are group mean fold induction over the averaged group baseline of the unstimulated PBMC control.

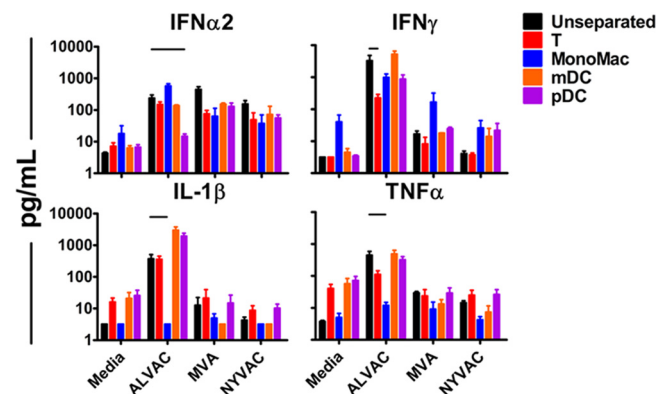


FIG 4 Cytokine and chemokine responses elicited by ALVAC in human PBMC depleted of various cell populations. Fresh whole human PBMC ($n = 2$ to 6/group) were depleted of the indicated PBMC populations, and depletion was confirmed by flow cytometry (data not shown). Unseparated and depleted cell populations were stimulated at an MOI of 10 PFU/cell with ALVAC, MVA, or NYVAC, and cytokine and chemokine responses were measured by Luminex assays at 24 h postinfection. The data shown are means and standard errors of the means. Lines indicate $P < 0.01$ by Kruskal-Wallis tests.

clinically relevant poxvirus vectors. Additional studies are required to evaluate the correlation between these different innate signatures and subsequent adaptive immune responses and protective efficacy.

A possible source of the observed differences in innate stimulatory phenotypes among ALVAC, MVA, and NYVAC involves their different arsenals of immune regulatory genes. Poxviruses possess a wide array of proteins that serve to block host antiviral immune responses (14–18). Importantly, ALVAC differs significantly from both vaccinia virus-derived vectors MVA and NYVAC in terms of extensive phylogenetic divergence, as well as genome size (approximately 365 kbp versus approximately 178 kbp, respectively) (14, 19) and the number of open reading frame (ORFs) (7, 19, 20). As stated above, the ALVAC, MVA, and NYVAC vectors are replication incompetent when used *in vivo* and many of ALVAC’s ORFs may not be functional in mammalian cells, as evidenced by the inability of avipoxviruses to replicate in mammalian cells (21–23). Previous reports have indicated increased innate immune gene upregulation following MVA and NYVAC infection relative to vaccinia virus Western Reserve infection in HeLa cells (24–26), highlighting that attenuation of these vectors by the removal of viral immune regulatory genes confers a stronger innate stimulatory phenotype. These studies reported that MVA elicited a stronger IFN-stimulatory phenotype and

NYVAC induced a more proinflammatory phenotype, congruent with our results in the present study. Differences in immune gene upregulation and induced immune phenotypes were further seen following MVA and NYVAC infections *in vitro* and *in vivo* (27–30). Further studies showed increased innate stimulation by these vectors following expanded deletion of their immune regulatory gene repertoire, highlighting the role of the immune gene repertoire of poxvirus vectors in their innate stimulatory phenotypes (31–34). However, far less is known about the innate immune profile of ALVAC and its immune regulatory genes (35). Our findings suggest that ALVAC stimulation of innate immunity requires several PBMC cellular subsets, which is consistent with prior studies that have suggested that monocyte tropism is a key difference in innate triggering by ALVAC, MVA, and NYVAC (36). The unique innate immune profile of ALVAC may also relate to its avian origin, as opposed to MVA and NYVAC. The molecular basis of this innate stimulatory phenotype and whether it is unique to ALVAC or extends to other avipoxviruses warrant further study.

Our results indicate that the innate immune profiles elicited by the leading poxvirus vaccine vectors are different, suggesting potentially important biological differences. In particular, ALVAC induced a unique proinflammatory cytokine and chemokine response following the vaccination of rhesus monkeys and infection of human PBMC. The extent to which these properties are advantageous to vaccines, however, remains to be determined. We previously reported substantial differences in innate immune profiles among various serotypes of adenovirus vectors (11). Taken together, these data suggest that vectors from the same family can differ markedly in their biological and innate stimulatory properties, which may potentially impact the resultant adaptive immune responses and protective efficacy of vector-based vaccines.

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