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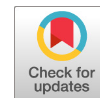
VACCINES AND ANTIVIRAL AGENTS



Distinct Roles of Vaccinia Virus NF-κB Inhibitor Proteins A52, B15, and K7 in the Immune Response

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ABSTRACT Poxviruses use a complex strategy to escape immune control, by expressing immunomodulatory proteins that could limit their use as vaccine vectors. To test the role of poxvirus NF-κB pathway inhibitors A52, B15, and K7 in immunity, we deleted their genes in an NYVAC (New York vaccinia virus) strain that expresses HIV-1 clade C antigens. After infection of mice, ablation of the *A52R*, *B15R*, and *K7R* genes increased dendritic cell, natural killer cell, and neutrophil migration as well as chemokine/cytokine expression. Revertant viruses with these genes confirmed their role in inhibiting the innate immune system. To different extents, enhanced innate immune responses correlated with increased HIV Pol- and Gag-specific polyfunctional CD8 T cell and HIV Env-specific IgG responses induced by single-, double-, and triple-deletion mutants. These poxvirus proteins thus influence innate and adaptive cell-mediated and humoral immunity, and their ablation offers alternatives for design of vaccine vectors that regulate immune responses distinctly.

IMPORTANCE Poxvirus vectors are used in clinical trials as candidate vaccines for several pathogens, yet how these vectors influence the immune system is unknown. We developed distinct poxvirus vectors that express heterologous antigens but lack different inhibitors of the central host-cell signaling pathway. Using mice, we studied the capacity of these viruses to induce innate and adaptive immune responses and showed that these vectors can distinctly regulate the magnitude and quality of these responses. These findings provide important insights into the mechanism of poxvirus-induced immune response and alternative strategies for vaccine vector design.

KEYWORDS NF-κB, NYVAC, T cell immunity, adaptive immunity, human immunodeficiency virus, immunomodulation, innate immunity, poxvirus, vaccines, vaccinia virus

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Several vaccine strategies have been developed to improve immune responses to heterologous antigens expressed by attenuated poxvirus vectors (1). Given the limited effectiveness of the ALVAC poxvirus vector in the RV144 phase III HIV/AIDS clinical trial (2), the need remains to improve poxvirus vector capacity as an immunogen, to increase protection levels, and to understand how innate and adaptive immune responses can be regulated.

The attenuated poxvirus strain NYVAC (New York vaccinia virus) has been used as a vaccine vector in HIV clinical trials (3, 4). Studies in nonhuman primates showed that NYVAC expressing Env and/or the Gag-Pol-Nef (GPN) clade C HIV antigens elicited a balanced CD4/CD8 T cell response (5) or robust T cell immunity (6). In clinical trials in healthy volunteers and in chronically HIV-infected patients, NYVAC showed clear immunogenic potential to induce expansion of preexisting T cell responses as well as the appearance of newly detected polyfunctional CD8 T cell responses (7).

Deletion of vaccinia virus (VACV) immunomodulatory genes that encode inhibitors of the Toll-like receptor (TLR) pathway is a common vaccine optimization strategy (8–10). VACV binding induces TLR2 and TLR4 homo- or heterodimerization (11, 12); this is followed by recruitment of Toll/interleukin-1 receptor (IL-1R) (TIR) domain-containing adaptor proteins such as myeloid differentiation factor 88 (MyD88) and MyD88-adaptor-like (MAL) or TIR domain-containing adapter inducing beta interferon (IFN-β) (TRIF) and TRIF-related adaptor molecule (TRAM). MyD88 mediates activation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) by phosphorylation of IL-1R-associated kinases (IRAK) 1, 2, and 4; TRIF interacts directly with TRAF6. TRAF6 recruits transforming growth factor beta (TGF-β)-activated kinase (TAK1) as well as TAK1-binding proteins 1 (TAB1) and 2 (TAB2) to phosphorylate TAK1 and activate the IκB kinase (IKK) complex (13).

A52, B15 (corresponding to Western Reserve strain B14), and K7 are VACV inhibitors of the TLR pathway (14). A52 and K7 block IRAK2 and TRAF6 activation (15, 16), and B15 inhibits IκBα phosphorylation and proteasome degradation (17), thus avoiding release of the NF-κB complex into the cell nucleus. NYVAC infection limits NF-κB binding to the κB site and transcription of proinflammatory cytokine and chemokine genes (18).

Cytokine and chemokine proinflammatory signals are involved in recruiting several cell types that act as the first cell line of defense in the innate immune response (19), including monocytes, dendritic cells (DC), natural killer (NK) cells, B cells, and neutrophils, which generate adaptive immunity (20). Distinct poxvirus vectors induce substantial differences in chemokine and cytokine profiles (21), which influence the adaptive immune responses. Whereas DCs and neutrophils transport VACV antigens to lymphoid organs and directly induce antigen-specific T cell responses (22, 23), NK cells can generate the environment necessary to induce recruitment of activated T cells (20) or cross talk with DCs for the generation of antigen-specific T cell immunity (24).

We prepared several NYVAC deletion mutants that express HIV-1 Env and GPN clade C antigens (NYVAC-C), but lack the *A52R*, *K7R*, and *B15R* genes that encode TLR NF-κB inhibitors. The effect of their concomitant ablation on increased neutrophil migration and HIV-specific CD8 T cell responses has been studied (18). Here we used NYVAC-C (18) to define the specific role of single, double, and triple deletion of these genes in innate and adaptive HIV-specific cell-mediated and humoral responses. We show that deletion of these genes increases cytokine/chemokine secretion and innate immune responses at the infection site. This enhanced innate immune response correlates with increased HIV-specific adaptive cell-mediated and humoral immunity.

RESULTS

Deletion of NF-κB inhibitory proteins in NYVAC leads to increased innate immune responses. To define the immunomodulatory role of the VACV NF-κB inhibitory A52, K7, and B15 proteins, we used mutants with single, double, and triple deletions of these genes on a backbone NYVAC-C vector expressing HIV-1 clade C antigens Env (gp120) as a cell-released product and Gag-Pol-Nef (GPN) as an intracellular polyprotein (25). BALB/c mice were infected by intraperitoneal (i.p.) injection of 10^7 PFU of the sucrose-purified NYVAC-C and NYVAC-C deletion mutants NYVAC-C Δ*B15R*, NYVAC-C Δ*A52R*, NYVAC-C Δ*K7R*, NYVAC-C Δ*B15R* Δ*K7R*, NYVAC-C Δ*A52R* Δ*B15R*, NYVAC-C Δ*A52R* Δ*K7R*, and NYVAC-C Δ*A52R* Δ*B15R* Δ*K7R* (here termed the NYVAC-C Δ3 or triple-deletion mutant). Reverse transcription-PCR (RT-PCR) of BSC-40 cells infected with the different viruses confirmed the lack of *A52R*, *K7R*, and *B15R* gene transcription following gene deletion (Fig. 1A to C).

Peritoneal exudate cells (PECs) were collected 6 h postinfection to study the innate immune response. Mice infected with NYVAC-C Δ*A52R*, NYVAC-C Δ*A52R* Δ*K7R*, or NYVAC-C Δ*A52R* Δ*B15R* showed an increase in the percentages of CD11c$^+$ major histocompatibility complex class II (MHC-II)$^+$ DCs compared to NYVAC-C-infected mice (Fig. 2A). Absolute DC numbers in these groups were significantly enhanced (Fig. 2B). There were no differences in DC migration between mice that received *K7R* or *B15R* single or combined deletion and mice that received the parental virus (Fig. 2B). Mice

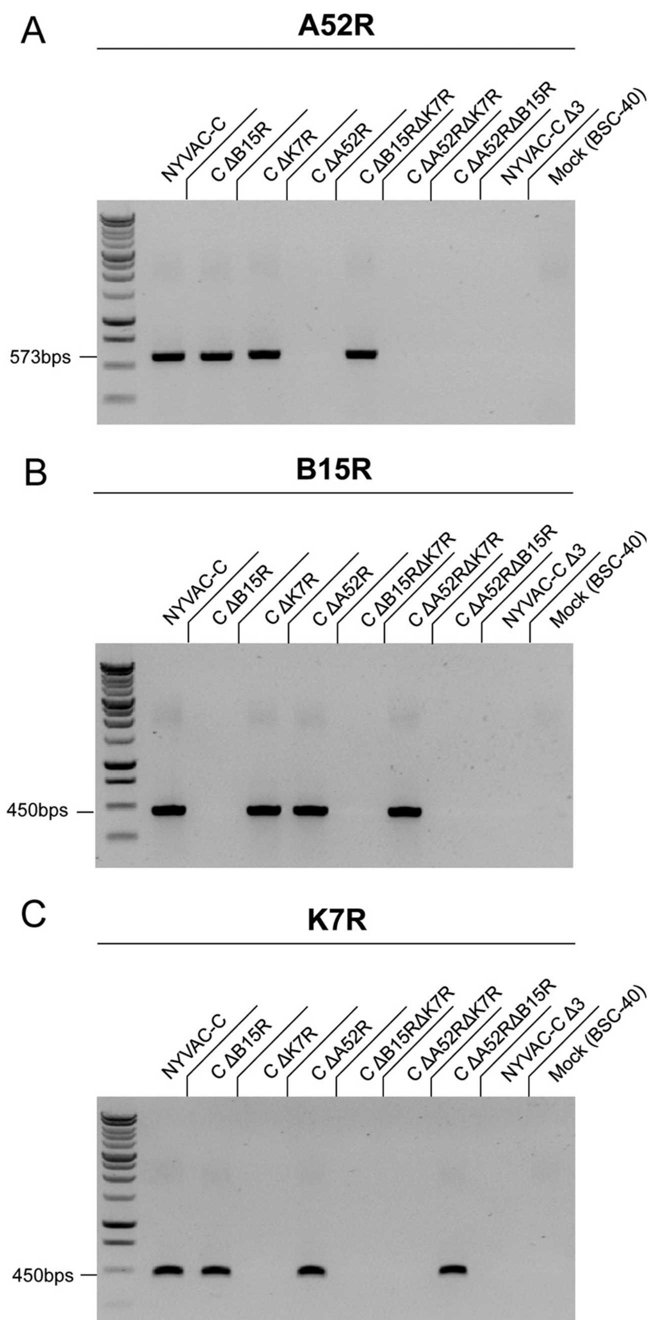

FIG 1 Lack of transcription of the *A52R*, *B15R*, and *K7R* genes in NYVAC deletion mutant-infected cells. BSC-40 cells were infected with NYVAC-C and NYVAC deletion mutant viruses (1 PFU per cell, 24 h). RT-PCR analysis of infected cells confirms the deletion of the *A52R* (A), *B15R* (B), and *K7R* (C) genes by loss of RNA expression.

injected with NYVAC-C Δ3 showed a significant increase in DC numbers compared to those that received the NYVAC-C Δ*A52R* Δ*K7R* or NYVAC-C Δ*B15R* Δ*K7R* double-deletion mutants (Fig. 2B).

Compared to the parental and single-deletion mutants, NYVAC-C Δ*A52R* Δ*B15R* and NYVAC-C Δ*B15R* Δ*K7R* showed an increase in the percentages of neutrophils in the peritoneal cavity (Fig. 2C). NYVAC-C Δ3 further enhanced neutrophil migration (Fig. 2C) and significantly increased absolute neutrophil numbers compared to all other groups (Fig. 2D).

The percentages and absolute numbers of NK cells in NYVAC-C Δ3-injected mice were also higher than in NYVAC-C-, NYVAC-C Δ*A52R*- or NYVAC-C Δ*K7R*-injected mice

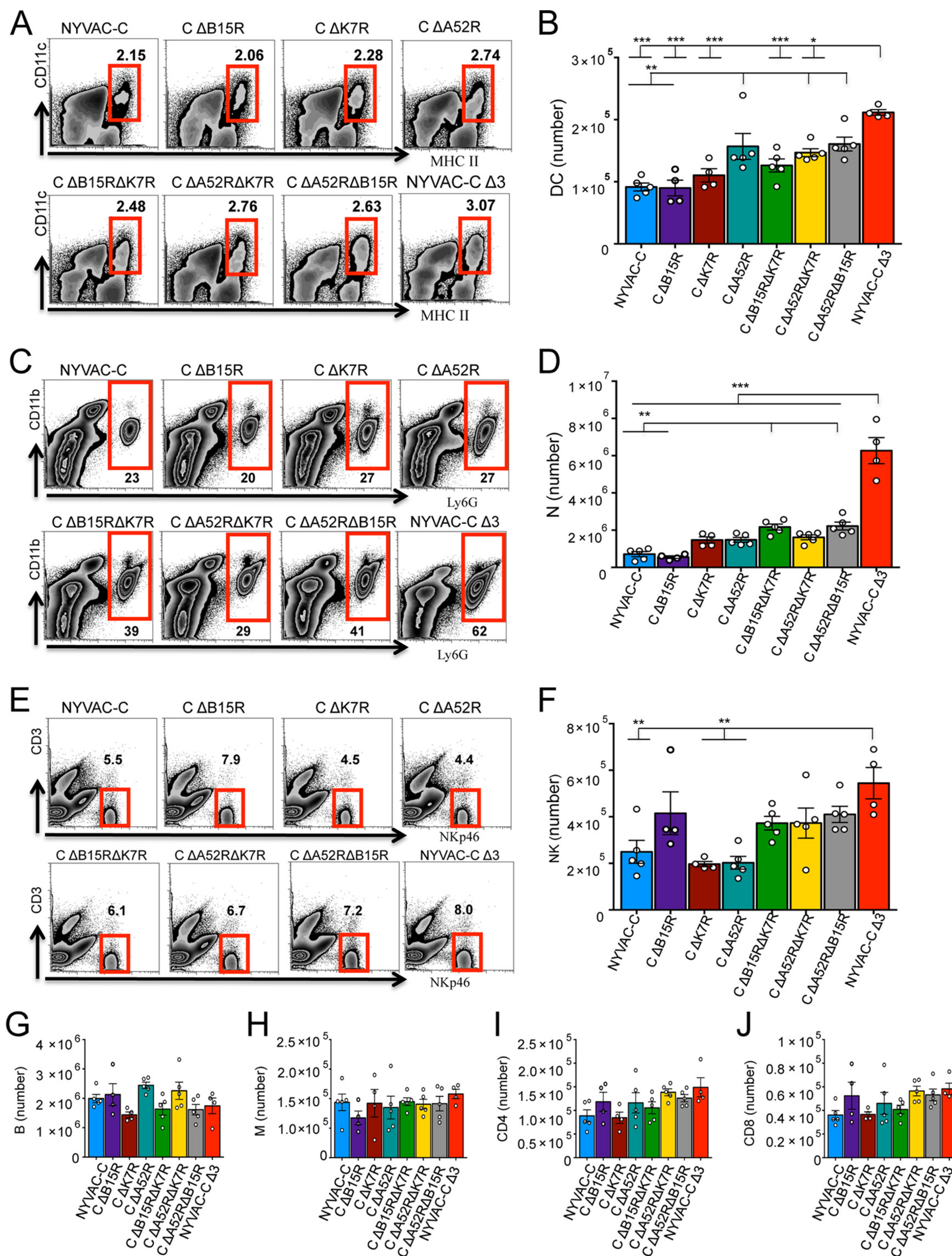

FIG 2 Deletion of the *A52R*, *B15R*, and *K7R* genes influences peritoneal cell migration. Shown are the percentages and absolute numbers of CD11c+ MHC-II+ dendritic cells (DCs) (A and B), Ly6G+ CD11b+ neutrophils (N) (C and D), NKp46+ CD3− natural killer cells (NK) (E and F), CD19+ B cells (B) (G), F4/80low CD11blow monocytes (M) (H), CD4+ CD3+ T cells (CD4) (I), and CD8+ CD3+ T cells (CD8) (J) in the peritoneal cavity of BALB/c mice at 6 h postinjection of 10^7 PFU of NYVAC-C or the NYVAC-C deletion mutants. Graphs show the mean ± standard error of the mean (SEM); each point represents an individual mouse. Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 2E and F). There were no significant differences between single-deletion mutants and NYVAC-C, between double- and single-deletion mutants, or between triple- and double-deletion mutants in total numbers of monocytes or B cells or CD4 and CD8 T cells (Fig. 2G to J).

These data indicate that *A52R* single gene deletion partially influences the innate immune response, as indicated by DC migration. Double gene deletion had a clear effect on DC and neutrophil migration, and concomitant *A52R*, *B15R*, and *K7R* deletion increased DC, neutrophil, and to some extent, NK cell migration.

To determine whether this altered innate immune response correlates with a change in cytokine/chemokine secretion at the infection site, we analyzed peritoneal exudates (PEs) of 6-h NYVAC-C- and NYVAC-C deletion mutant-infected mice. IL-6, CXCL1 (KC), CXCL2 (MIP-2), CXCL9 (MIG), CXCL10 (IP-10), CCL2 (MCP-1), CCL3/4 (MIP-1α/β), and CCL5 (RANTES) cytokines/chemokines are essential for recruitment of several cell types (20, 26). The proinflammatory cytokine IL-6 and the chemokines CXCL1 and CXCL2, which mainly induce neutrophil migration (27, 28), were significantly overexpressed in PEs of NYVAC-C Δ3-injected mice compared to those of mice injected with the other deletion mutants (Fig. 3A to C). CCL3 is involved in DC migration (29), whereas CXCL9, CXCL10, and to some extent CCL2 induce NK cell migration (30, 31). While CCL2 increased significantly after i.p. injection of NYVAC-C Δ3 compared to the other viruses (Fig. 3D), there was no notable difference in expression of CCL3, CXCL10, and CXCL9 in these groups (Fig. 3E to G).

The data suggest that infection by the triple-deletion mutant induced a chemokine pattern that influences mainly neutrophil migration and partially affects DC and NK cell migration. The significant cytokine/chemokine increase in NYVAC-C Δ3-injected mice correlated with enhanced neutrophil migration in these mice.

To define the distinct roles of these immunomodulatory genes in inducing innate immune responses, we used NYVAC-C Δ3 *K7R*-rev virus (18) and generated revertant NYVAC-C Δ3 *A52R*-rev and NYVAC-C Δ3 *B15R*-rev viruses, in which the *B15R* and *A52R* genes were reinserted at the hemagglutinin locus (*HA*); expression was confirmed by RT-PCR (Fig. 4A and B). The percentage of neutrophils was lower in the peritoneal cavities of all revertant virus-injected mice compared to triple-deletion mutant-infected mice (Fig. 5A). Absolute neutrophil numbers in revertant virus-infected mice were significantly lower than those in mice injected with NYVAC-C Δ3 (Fig. 5B). DC percentages were similar for all virus-injected mice (Fig. 5C), and absolute DC numbers were only significantly lower between NYVAC-C Δ3 *K7R*-rev- and triple-deletion mutant-infected mice (Fig. 5D). The percentages (Fig. 5E) and absolute numbers (Fig. 5F) of NK cells showed no differences between triple-deletion mutant- and revertant virus-treated mice. Again, no differences were found in absolute numbers of B cells (Fig. 5G), monocytes (Fig. 5H), or CD4 (Fig. 5I) or CD8 T cells (Fig. 5J) among the virus-injected mice. Peritoneal concentrations of IL-6, CCL2, and CXCL2 (Fig. 5K to M) were significantly lower after infection with revertant viruses compared to NYVAC-C Δ3. We found no marked differences in CXCL1 levels between NYVAC-C Δ3- and revertant virus-injected mice (Fig. 5N). These data show a correlation between chemokine levels and neutrophil migration and indicate that these VACV proteins have a central role in inhibiting innate immune responses.

Deletion of NF-κB inhibitory proteins enhances HIV-specific T cell and humoral responses. To study the ability of NYVAC-C deletion mutants to induce HIV-specific adaptive cellular responses, we tested a heterologous DNA prime/poxvirus boost immunization regimen in BALB/c mice, a protocol widely used to enhance immune responses to HIV antigens (18, 25). At 11 days after the last immunization, splenocytes from NYVAC-C- or NYVAC-C deletion mutant-infected mice were stimulated with HIV-1 Pol-1, Pol-2, or Gag-Pool (representing 60 peptides of Gag-1 plus 61 peptides of Gag-2) to study HIV-1-specific CD8 T cell responses (32). The magnitude of CD8 T cell responses was measured as the expression of IL-2 and/or IFN-γ and/or TNF-α and/or CD107a activation markers. NYVAC-C Δ3 and NYVAC-C Δ*A52R* Δ*B15R* induced the largest number of TNF-α^+ CD107a$^+$, IFN-γ^+ TNF-α^+, or IFN-γ^+ CD107a$^+$ double-positive cells (Fig. 6A). Only a small percentage of CD8 T cells expressed IL-2 (Fig. 6A). Of the single-deletion mutants, only NYVAC-C Δ*A52R* significantly enhanced adaptive Pol-1 CD8 T cell responses compared to the parental NYVAC-C virus (Fig. 6B). Compared to the single-deletion mutants, NYVAC-C Δ*B15R* Δ*K7R* and NYVAC-C Δ*A52R* Δ*K7R* signifi-

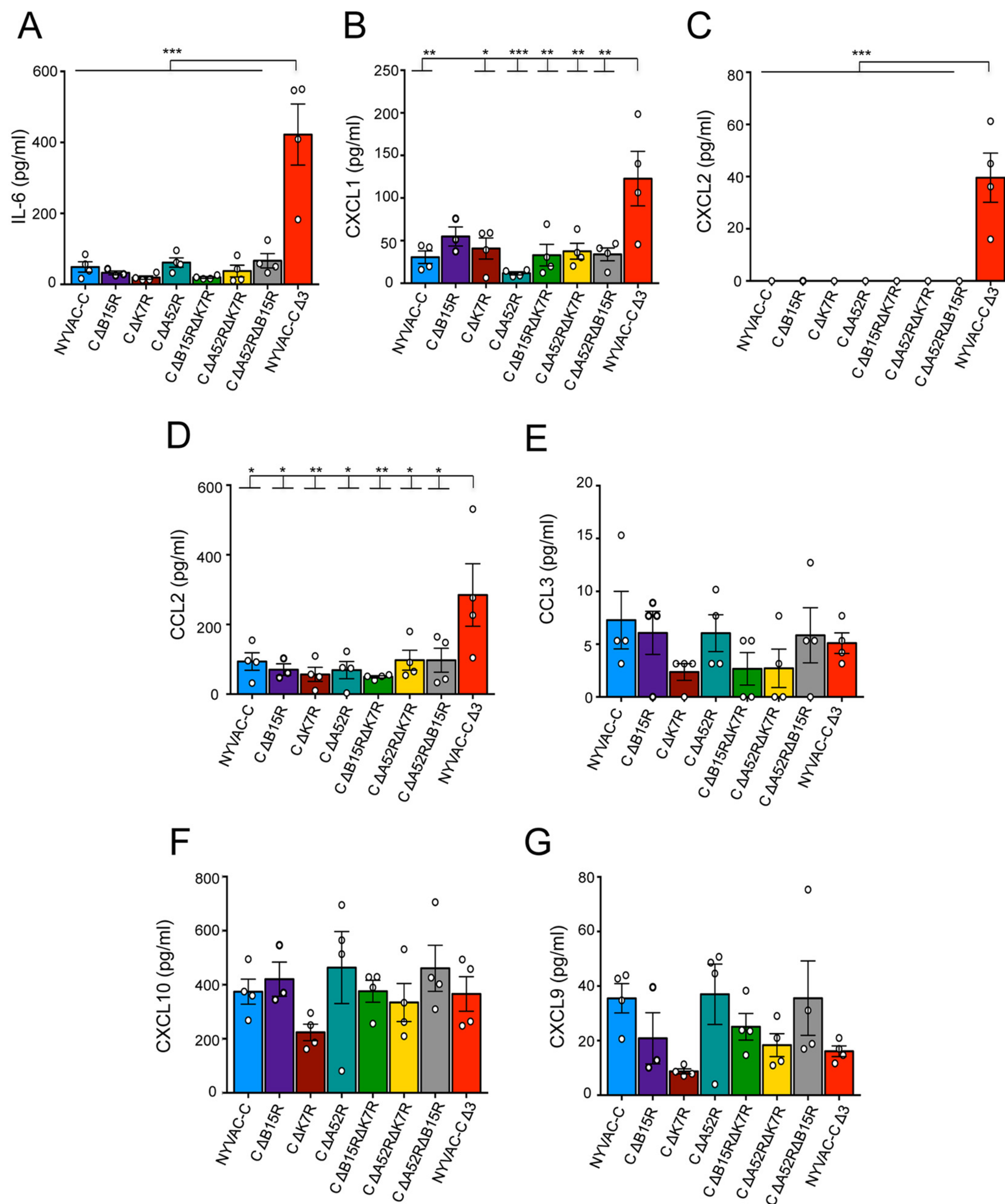

FIG 3 Deletion of the *A52R*, *B15R*, and *K7R* genes affects cytokine/chemokine levels. Shown are concentrations of cytokine IL-6 (A) and chemokines CXCL1 (B), CXCL2 (C), CCL2 (D), CCL3 (E), CXCL10 (F), and CXCL9 (G) at 6 h postinfection in the peritoneal cavity of NYVAC-C- or NYVAC-C deletion mutant-injected mice. Graphs show the mean ± SEM; each point represents an individual mouse. Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cantly increased Gag-Pool and Pol-2 responses, respectively (Fig. 6C and D). NYVAC-C Δ3 and NYVAC-C Δ*A52R* Δ*B15R* elicited the strongest CD8 T cell responses to the three HIV antigens (Fig. 6B to D). Only in the case of Pol-2, did the triple-deletion mutant significantly increase CD8 T cell responses compared to NYVAC-C Δ*A52R* Δ*B15R* (Fig. 6D). The quality of the Gag and Pol responses, defined as cytokine production and cytotoxic potential, showed that the NYVAC-C Δ*A52R* Δ*B15R* and NYVAC-C Δ*A52R* Δ*K7R* double-deletion mutants and NYVAC-C Δ3 triple-deletion mutant induced a marked increase in the cytotoxic T lymphocyte (CTL) polyfunctional profile compared to the single-deletion mutant NYVAC-C Δ*A52R* (Fig. 6E; pie charts). The CD8 T cell subset that produced IFN-γ, TNF-α, and CD107a was the most representative population induced with HIV peptides (Fig. 6E; bar graph). This CD8 T cell subset marked the difference in

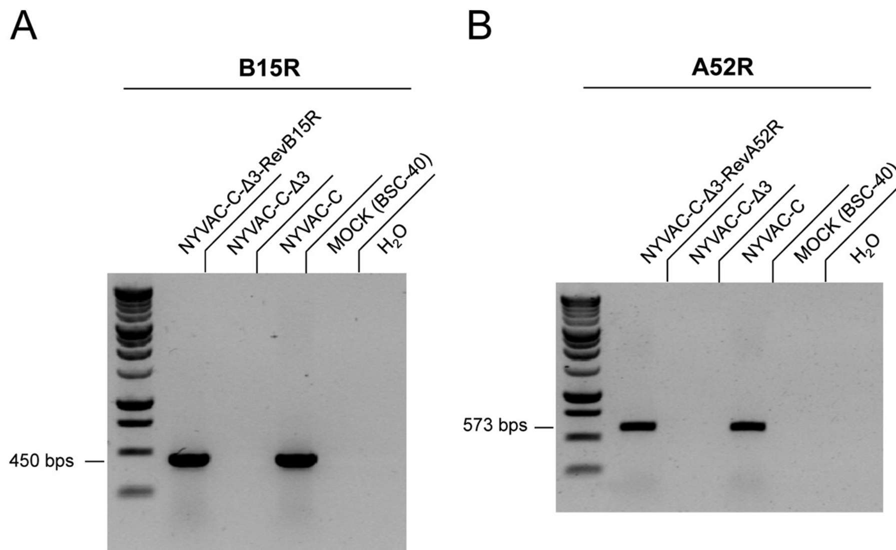

FIG 4 Characterization of the NYVAC-C-Δ3 revertant viruses. RT-PCR analysis of RNA from BSC-40 cells infected with the NYVAC-C-Δ3 *B15R*-rev and NYVAC-C-Δ3 *A52R*-rev revertants (Rev) (1 PFU per cell, 24 h) confirms the correct *B15R* (A) and *A52R* (B) gene reinsertion and transcription.

the CTL polyfunctional profiles among the virus-infected mice. These results indicate that the double- and triple-deletion mutants enhanced the magnitude and polyfunctional profile of specific CD8 T cells to HIV-1 Gag and Pol intracellular antigens.

To determine whether the altered innate immune response correlates with changes in HIV-specific adaptive humoral responses, we measured Env-specific IgG antibodies in the sera of mice immunized as described above. Compared to the parental virus, NYVAC-C Δ*A52R* was the only single-deletion mutant that significantly increased Env-specific total IgG and IgG1 antibody titers (Fig. 7A and B). Double-deletion mutant-infected mice showed significantly higher Env-specific total IgG, IgG1, and IgG3 antibody levels compared to those infected with NYVAC-C and NYVAC-C single-deletion mutants (Fig. 7A to C). NYVAC-C Δ*A52R* Δ*B15R* significantly increased Env-specific total IgG, IgG1, and IgG3 responses compared to the other double-deletion mutants (Fig. 7A to C). Mice infected with NYVAC-C Δ*A52R* Δ*B15R* and NYVAC-C Δ3 showed similar Env-specific IgG1 and IgG3 antibody titers (Fig. 7B and C). Compared to NYVAC-C Δ*A52R* Δ*B15R*, the triple-deletion mutant only significantly increased the total IgG response (Fig. 7A).

These data show that *A52R* single-gene deletion partially influences HIV-specific cell-mediated and humoral immune responses, represented by Pol-1 CD8 T cell responses and by Env-specific total IgG and IgG1 levels. Of the double-deletion mutants, NYVAC-C Δ*A52R* Δ*B15R* induced the highest HIV-specific CD8 T cell and IgG responses. Additional deletion of *K7R* in NYVAC-C Δ*A52R* Δ*B15R* had a further positive effect on Pol-2- and Env-specific total IgG immune responses.

DISCUSSION

A52, K7, and B15 vaccinia virus proteins, members of the B cell lymphoma-2 family (Bcl-2) (33, 34), have an important NF-κB inhibitory role (18) and thus induce suppression and evasion of host immune responses (14). During infection, A52 and K7 interact with TRAF6 and inhibit its kinase activity (15, 16), whereas B15 binds the IKK complex to inhibit IκBα phosphorylation and degradation (17). Using single-, double- and triple-deletion mutants, we showed that combined deletion of the *A52R*, *K7R*, and *B15R* genes efficiently triggers innate immune responses. With the generation of revertants NYVAC-C Δ3 *K7R*-rev, NYVAC-C Δ3 *A52R*-rev, and NYVAC-C Δ3 *B15R*-rev, we also determined that one of these inhibitory molecules is enough to reduce chemokine release and cell migration to infection sites. A46 and C49 are other NYVAC NF-κB inhibitors (35, 36) whose contribution to induction of innate immune responses might be better determined by removing these

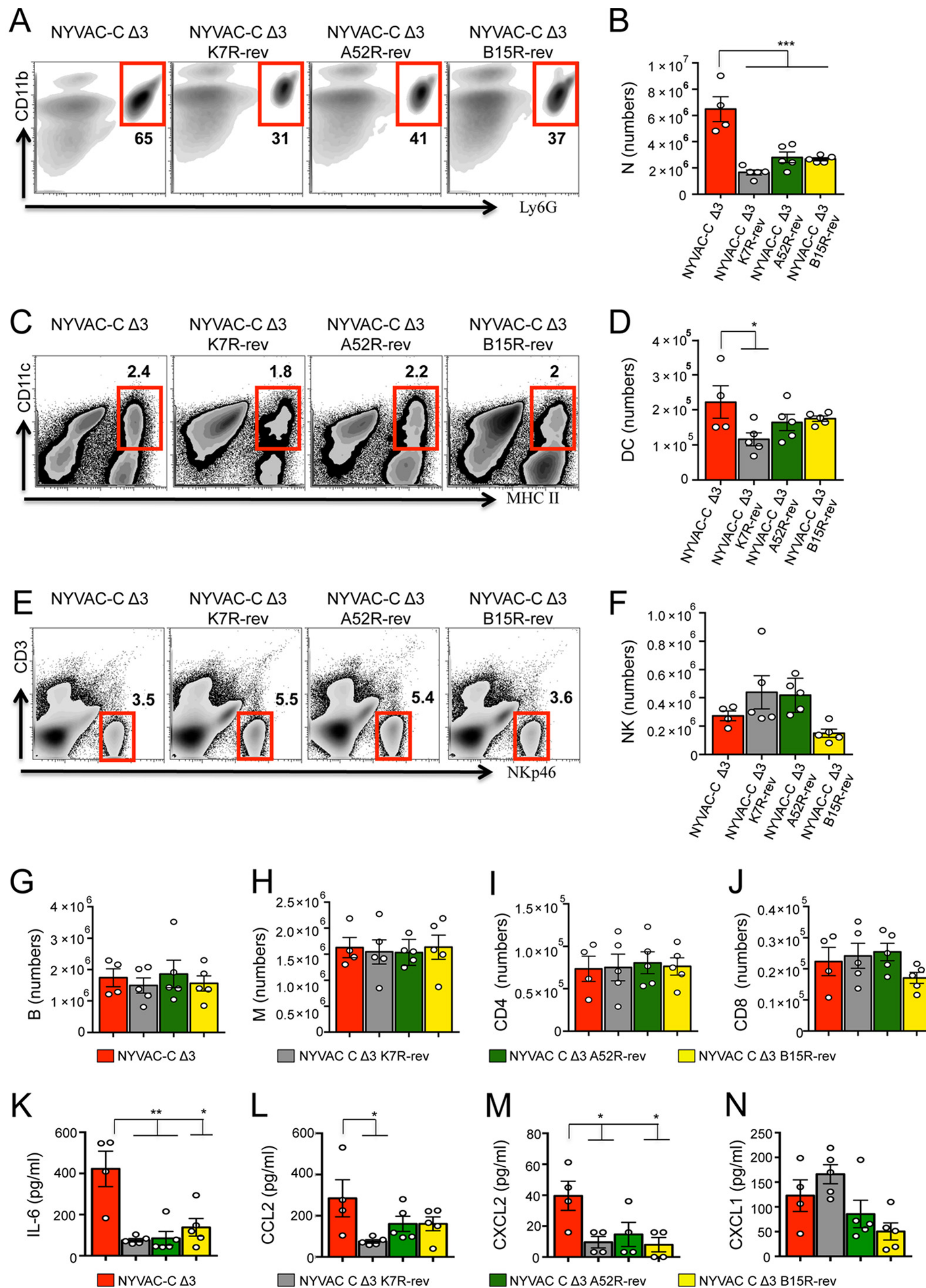

FIG 5 Revertant viruses reduce cell migration and cytokine/chemokine levels. Shown are the percentages and absolute numbers of Ly6G$^+$ CD11b$^+$ neutrophils (N) (A and B), CD11c$^+$ MHCII$^+$ DCs (C and D), NKp46$^+$ CD3$^-$ NK cells (E and F), CD19$^+$ B cells (B) (G), F4/80^{low} CD11b^{low} monocytes (M) (H), CD4$^+$ CD3$^+$ T cells (CD4) (I) and CD8$^+$ CD3$^+$ T cells (CD8) (J) in the peritoneal cavity of BALB/c mice at 6 h postinjection of 10^7 PFU of NYVAC-C triple-deletion mutants and revertant viruses. (K to N) Concentrations of IL-6 (K), CCL2 (L), CXCL2 (M), and CXCL1 (N) in the peritoneal cavity of infected mice. Data are representative of two independent experiments. Graphs show the mean ± SEM; each point represents an individual mouse. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

genes from the backbone of the NYVAC triple-deletion mutant, followed by testing under conditions of strong NF-κB activation and cell migration.

Effective antigen presentation by DCs is NF-κB dependent (37), and direct DC stimulation of CD8/CD4 T and B cells induces cytokine and antibody secretion (38, 39).

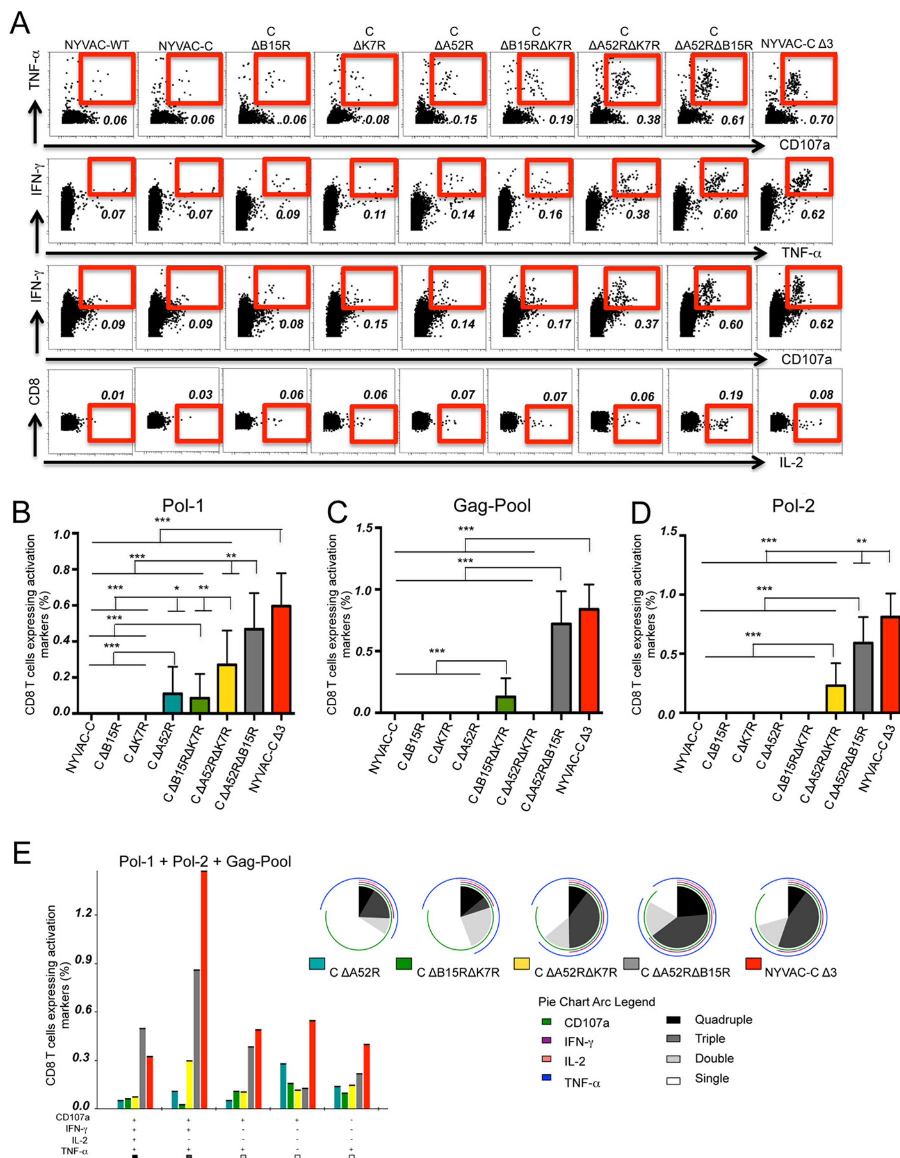

FIG 6 Deletion of the *A52R*, *B15R*, and *K7R* genes influences adaptive HIV-specific CD8 T cell immune responses. Shown are the vaccine-induced HIV-specific CD8 T cell response in mice ($n = 4$/group) immunized following a heterologous DNA prime/virus boost regimen (10^7 PFU of NYVAC-C or NYVAC-C deletion mutants). (A) Percentages of TNF-α^+ CD107a$^+$, IFN-γ^+ TNF-α^+, IFN-γ^+ CD107a$^+$ double-positive and IL-2 single-positive T cells. The total value (magnitude) is the sum of percentages of CD8 T cells that express IFN-γ and/or TNF-α and/or IL-2 and/or CD107a, measured by intracellular cytokine staining (ICS). Nonspecific responses of mice infected with control NYVAC-WT were subtracted from the total magnitude. (B to D) Magnitude of Pol-1 (B)-, Gag-Pool (C)-, or Pol-2 (D)-specific CD8 T cell responses. Graphs show the mean ± confidence interval (CI). (E) Functional profile of adaptive Gag-Pol-specific CD8 T cells. Combinations of responses (*x* axis) and percentages of functionally distinct cell subsets (*y* axis) are shown in the bar graph. Responses are grouped and color coded based on the number of functions. Pie chart colors indicate the percentage of cytokine-producing cells based on the number of functions (inside) and the different activation markers (outside). Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

We show that A52 is partially involved in DC evasion and suggest that DCs recruited after infection are involved in HIV-specific CD8 T cell and IgG responses. Neutrophils cross-prime naive T cells and induce adaptive immune responses (40, 41). *A52R*, *B15R*, and *K7R* gene deletion enhances chemokines that can directly induce neutrophil migration, and thus HIV-specific CD8 T cell and IgG responses, which supports neutrophil mediation of HIV immune responses (18). NK cells also mediate adaptive immune responses (42), and A52, B15, and K7 together are needed to reduce NK cell migration

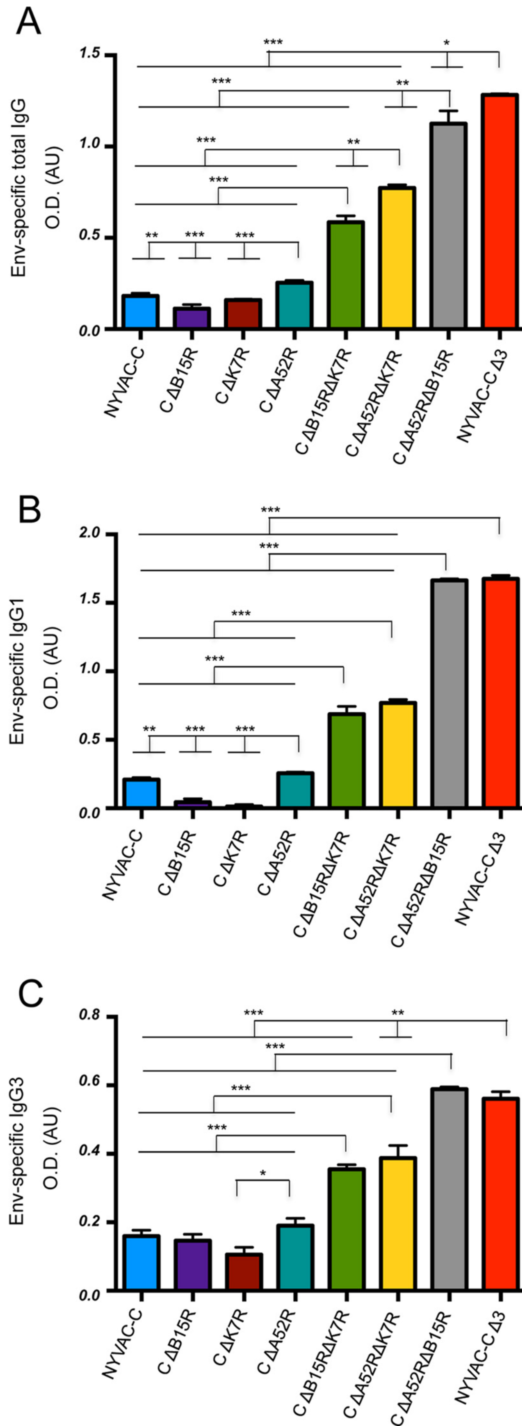

FIG 7 Deletion of the *A52R*, *B15R*, and *K7R* genes affects humoral Env-specific IgG immune responses. Shown are vaccine-induced HIV-specific IgG responses in mice (n = 4/group) immunized following a heterologous DNA prime/virus boost regimen (10^7 PFU of NYVAC-C or NYVAC-C deletion mutants). (A to C) Levels of Env-specific total IgG (A), IgG1 (B), and IgG3 (C) antibodies in serum of mice (n = 4/group) 11 days postinfection with NYVAC-C or NYVAC-C deletion mutants. Data are represented as optical density (OD) in arbitrary units (AU). Nonspecific responses of mice infected with control NYVAC-WT were subtracted from total values. Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

after NYVAC infection. Ablation of their genes is sufficient to increase HIV-specific CD8 T cells and IgG responses, suggesting that NK cells also mediate these antigen-specific immune responses. Our results indicate that the A52, B15, and K7 VACV proteins influence immune responses differently (Table 1), which suggests new approaches to more efficient poxvirus vaccine design.

TABLE 1 Immune response levels induced by *A52R*, *B15R*, and *K7R* deletion mutants

	Immune response[a]								
				CD8 cells			Env-specific IgG		
Virus mutation(s)	DCs	NK cells	Neutrophils	Pol-1	Pol-2	Gag	Total IgG	IgG1	IgG3
Δ*B15R*									
Δ*K7R*									
Δ*A52R*	++			+++			++	++	
Δ*B15R* Δ*K7R*			++	+++		+++	+++	+++	+++
Δ*A52R* Δ*K7R*	++			++++	+++		++++	+++	+++
Δ*A52R* Δ*B15R*	++		++	+++++	++++	+++++	+++++	+++++	++++
Δ*A52R* Δ*B15R* Δ*K7R*	+++	++	+++	+++++	+++++	+++++	++++++	+++++	++++

[a]+ indicates a significant increase in innate, adaptive cell-mediated, or humoral immune responses of NYVAC-C deletion mutants compared to NYVAC-C.

Attenuated VACV vectors (NYVAC and MVA) are used as vaccine candidates for emerging infectious diseases and cancer in humans (43). Whereas distinct VACV vaccine approaches for HIV have yielded promising results in primates (44, 45), their effectiveness was limited in a human clinical trial (2), which prompted improvement of poxvirus vectors as HIV vaccine candidates. Immunomodulatory viral gene deletion is a strategy for VACV vectors to improve immunogenicity to HIV (46). Combined or single deletion of the *A52R*, *B15R*, and *K7R* genes might thus be relevant for the generation of new HIV-VACV vaccines that specifically modify innate immune responses and thus cellular or humoral adaptive immune responses.

The abilities of double-deletion and NYVAC-C Δ3 triple-deletion mutants to generate robust polyfunctional Gag- and Pol-specific CD8 T cell responses and Env-specific IgG antibodies are important immune properties for future vaccine applications. In the RV144 phase III clinical trial, effective vaccination correlated with humoral responses to Env, specifically human antibodies to the V_1/V_2 loops of HIV-1 gp120 (47). Here we determined that the combined deletion of the *A52R*, *B15R*, and *K7R* genes increased Env-specific total IgG, IgG1, and IgG3 responses compared to single deletions, which suggests that VACV vectors with NF-κB inhibitory protein ablation are encouraging therapeutic strategies.

Human HIV nonprogressor individuals preferentially maintain highly functional HIV-specific CD8 T cells (48). Gag and Pol, the most conserved HIV-1 proteins (49), can shift the CD8 T cell response from the variable Env epitope in the first years of HIV-1 infection (50). In chronically HIV-1-infected persons, a Gag CD8 T cell response correlates with lower HIV titers (51) and with decreased viremia in HIV-1-infected patients with suspension of antiretroviral therapy (50). A prophylactic vaccine that induces a Gag T cell response controls simian immunodeficiency virus (SIV) infection (52). Since the Gag/Pol-specific CD8 T cell response in double- and triple-deletion mutant-infected mice is robust and mainly polyfunctional compared to that in single-deletion mutants, these vectors could show promise for prophylactic and therapeutic treatment.

In summary, here we define the immune biological functions of three NF-κB inhibitors encoded by NYVAC and show that the vaccinia virus NYVAC deletion mutant lacking the *A52R*, *K7R*, and *B15R* genes is the most effective vector to trigger immunologically relevant markers of innate and adaptive immune responses. Based on these immunomodulatory properties, these vectors can be used for new HIV vaccine designs.

MATERIALS AND METHODS

Mice and injections. BALB/c mice (6 to 8 weeks old) were purchased from Harlan. In the DNA prime immunization protocol, mice received 100 μg DNA-C (50 μg pcDNA-CN54GP120 plus 50 μg pcDNA-CN54GPN) or 100 μg sham DNA-ϕ (100 μg pcDNA) by the intramuscular route. We purified plasmids using the Endofree plasmid megakit (Qiagen). After 2 weeks, mice were immunized intraperitoneally (i.p.) with 10^7 PFU of wild-type NYVAC (NYVAC-WT), NYVAC-C, and deletion mutants. Animal studies were approved by the Ethical Committee of Animal Experimentation (CEEA) of the Centro Nacional de Biotecnologia (CNB, Madrid, Spain) in accordance with national and international guidelines and the Royal Decree (RD 1201/2005) (permit no. 13013).

Cells. African green monkey kidney cells (BSC-40; American Type Culture Collection) and primary chicken embryo fibroblasts (CEFs [Intervet, Salamanca, Spain]) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) were used to grow the viruses.

Viruses. NYVAC-WT and NYVAC-C (Sanofi Pasteur), the NYVAC-C deletion mutants, and NYVAC-C Δ3 *K7R*-rev have been described previously (18, 25). NYVAC-C Δ3 *A52R*-rev and NYVAC-C Δ3 *B15R*-rev were constructed using the *A52R* and *B15R* VACV genes and the β-glucuronidase gene as a reporter gene. For the generation of revertant viruses, we used the virus hemagglutinin (HA) flanking regions to insert *K7R*, *B15R*, and *A52R* genes. BSC-40 cells were infected with 0.01 PFU/cell of NYVAC-C Δ3 (1 h) and transfected with VACV insertional plasmid vector pCAR-2/A52R or pCAR-2/B15R to insert the genes at the *HA* locus. Recombinant viruses were selected from progeny virus by consecutive rounds of plaque purification, as reported previously (53). All viruses were purified through two 36% (wt/vol) sucrose cushions, and their titers were determined by immunostaining plaque assay in BSC-40 cells (54).

Plasmids. The plasmid transfer vectors pCAR-2/A52R and pCAR-2/B15R were obtained by cloning the *A52R* and *B15R* sequence into the plasmid pCAR-2 (patent no. WO2007132052 A1 [55]). The NYVAC genome was used as the template to amplify the left flank, the repeated left flank, and the right flank of the genes. The following oligonucleotides were used: for *A52R*, forward, CGCGGATCCATGGACATAA AGATAG, and reverse, AAGGAAAAAAGCGGCCGCCTATGACATTTCCAC; and for *B15R*, forward, CTAGGCC TGGTACCCGGGGATGACAGCCAACTTTAGTACCC, and reverse, GGCCGCTCTAGAACTAGTGTCAATTCATACG CCGGAATATG. The resulting plasmids were confirmed by DNA sequence analysis.

PCR and RT-PCR. Viral DNA was extracted by the SDS-proteinase K-phenol method. We extracted total RNA using the RNeasy minikit (Qiagen) with RNase-free DNase I recombinant. First-strand cDNA was synthesized with oligo(dT)$_{12-18}$ primers using SuperScript III reverse transcriptase (both from Invitrogen), and cDNA was used for PCR amplification.

Cytokine/chemokine analysis. Peritoneal washes from phosphate-buffered saline (PBS)- or virus-injected mice were used to detect cytokines and chemokines in multiplex analysis using LuminexXMAP technology.

Peptides. The HIV-1 peptides Pol-1 (LVGPTPVNI) and Pol-2 (YYDPSKDLI) (32) were provided by the CNB-CSIC Proteomics Service. The HIV-1 Gag-Pool peptides (representing 60 peptides of Gag-1 plus 61 peptides of Gag-2) (25) were provided by the EuroVacc Foundation.

Flow cytometry. For intracellular cytokine staining (ICS), splenocytes were resuspended in RPMI 1640 with 10% FCS and 1 μg/ml Golgiplug (BD), monensin (eBioscience), and anti-CD107a (1D4B; BD), restimulated with peptides (6 h, 37°C, 5% CO_2), stained for surface markers with anti-CD3 (145-2C11), anti-CD4 (GK1.5), and anti-CD8 (53-6.7) (all from BD), fixed, permeabilized (Cytofix/Cytoperm kit; BD), and stained intracellularly with anti-IL-2 (JES6-5H4), anti-IFN-γ (XMG 1.2), and anti-TNF-α (MP6-X722) (all from BD). Peritoneal exudate cells (PECs) were stained with anti-Ly6G (1A8), anti-CD3 (145-2C11), anti-CD11b (M1/70), anti-CD19 (1D3), anti-MHC class II (1-A/1-E; 2G9), anti-CD4 (GK1.5), and anti-CD8 (53-6.7) (all from BD), anti-CD45 (30-F11; Biolegend), anti-CD11c (N418) and anti-F4/80 (BM8; both from eBioscience), and anti-NKp46 (29A1.4; BioLegend). Dead cells were stained using the violet LIVE/DEAD stain kit (Invitrogen). Cells were acquired using a Gallios (Beckman Coulter) flow cytometer, and analyses were performed with FlowJo software v.8.5.3 (Tree Star). Boolean combinations of single functional gates were used to determine the frequency of each response based on all combinations of activation markers.

Antibody measurement. Env-binding antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (25). Serum samples from mice were reacted in plates coated with 2 μg/ml recombinant CN54 gp120 purified protein (ARP683, HIV-1 CN54 gp120 clade C; EU Programme EVA).

Statistical analysis. For statistical analysis of CD8 T cell responses to HIV-1 antigens, we used an approach to correct measurements for medium response and allow calculation of confidence intervals and *P* values of hypothesis tests (53). Only antigen response values significantly larger than the corresponding RPMI values are shown. Background levels (splenocytes in RPMI) were subtracted from all values used. For distribution analysis and presentation, we used SPICE version 5.1 (http://exon.niaid.nih.gov). For statistical analysis of cell migration and cytokine/chemokine expression, one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* tests was applied. Student's *t* test was used for antibody analysis to establish the differences between two groups.

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