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Correlations Between Vaccinia-Specific Immune Responses Within a Cohort of Armed Forces Members

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Abstract

Widespread vaccination with vaccinia virus (VACV) resulted in the eradication of smallpox; however, the licensed VACV-containing vaccines are associated with adverse events (AEs), making them unsuitable for certain high-risk populations. A better understanding of the host immune response following smallpox vaccination could result in vaccines with similar immunogenicity profiles to pre-eradication vaccines with a lower incidence of AEs. To study the immune response to VACV, we recruited 1,076 armed forces members who had been vaccinated with one dose of Dryvax®. We measured multiple VACV-specific immune responses: neutralizing antibody titer, the level of 12 secreted cytokines in peripheral blood mononuclear cell (PBMC) cultures (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α, IFN-γ, IFN-α, IFN-β, and IL-18), and the number of IFN-γ- and $CD8^+$ IFN-γ-secreting cells. We analyzed these data to determine correlations between immune response measures. We detected a strong proinflammatory response in concert with a Th-1-like cytokine response pattern at a median time point of 15.3 mo following primary vaccination. We also detected correlations between neutralizing antibody titer and secreted IL-2, as well as secreted IFN-γ ($p = 0.009$ and $p = 0.0007$, respectively). We also detected strong correlations between the proinflammatory cytokines IL-1β, TNF-α, IL-6, and IL-12p40 ($p < 0.0001$). These results further advance our knowledge of vaccinia-specific cellular immune responses. Notably, vaccine-induced proinflammatory responses were not correlated with neutralizing antibody titers, suggesting that further attenuation to reduce inflammatory immune responses may result in decreased AEs without sacrificing VACV immunogenicity and population seropositivity.

Introduction

Widespread vaccination with vaccinia virus (VACV)-containing vaccines culminated in the eradication of smallpox (variola virus) in early 1980 (16). However, these highly effective vaccines are also associated with adverse events (AEs), including fever, headache, malaise, and in rare instances myopericarditis, post-vaccinal encephalitis, generalized vaccinia, and eczema vaccinatum, making them unsuitable for use in some populations, including infants, individuals who are immune compromised, and individuals with atopic dermatitis (11,17,21,31). A better understanding of immune responses to VACV could facilitate development of a new generation of vaccines that retain similar immunogenic profiles to the pre-eradication vaccines, but are modified to reduce the incidence of AEs following vaccination.

Multiple assays have been developed to characterize vaccinia-specific humoral and cell-mediated immune (CMI) responses.

The gold standard assay to assess humoral immunity is to quantify circulating vaccinia-specific neutralizing antibodies. An individual with a neutralizing antibody titer >1:32 is considered protected against a subsequent smallpox infection (15,17,18). No gold standard assay exists to quantify the vaccinia-specific CMI response, but evidence exists that a robust CMI response is needed to completely clear a smallpox infection (4,32). Thus, a successful immune response against VACV is the result of innate, humoral, and CMI responses, interacting iteratively and precisely with each other to induce a robust, persistent, and protective immune response.

We hypothesized that CMI responses to VACV could be correlated with neutralizing antibody titer, as well as the production of other cytokines known to influence the host

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immune response. The interplay between the humoral and CMI arms of adaptive immunity is complex; however, on a macro level we hypothesized that variations in individual immune measures could be correlated with each other to provide a high-level picture of the immune response interactions following primary vaccination with VACV.

In this study, we examined multiple immune measures (including innate, humoral, and cellular immune measures) following primary vaccination with VACV by conducting a population-based study on 1,076 individuals who had previously been vaccinated with a single dose of Dryvax® (18). Samples obtained from these individuals were used to quantify vaccinia-specific neutralizing antibody titer, secreted cytokines, and IFN-γ- and $CD8^+$ IFN-γ-secreting cells. Analyses were subsequently performed to examine correlations between all measures of the immune response.

Materials and Methods

Study subjects

As previously described, our study cohort was comprised of 1,076 healthy subjects who participated in either the Department of Health and Human Services civilian healthcare worker smallpox immunization program at the Mayo Clinic in Rochester, MN, or the smallpox immunization program at the Naval Health Research Center (NHRC) in San Diego, California (18). We enrolled 1,379 healthy subjects (age 18–40 y) in the study; however, 303 subjects were not eligible due to an incomplete blood sample, resulting in 1,076 successfully sampled subjects. All study subjects received a primary smallpox vaccination with a single dose of Dryvax (containing the New York City Board of Health [NYCBOH] strain of VACV) 1 mo to 4 y prior to enrollment, and had a documented vesicular lesion or "take" at the vaccination site. The administered Dryvax vaccine (Wyeth Laboratories, Inc., Marietta, PA) has a minimum concentration of 10^8 pock-forming units (PFU)/mL (27). The Institutional Review Board of the Mayo Clinic and NHRC approved the study, and written informed consent was obtained from each subject.

Isolation of peripheral blood mononuclear cells (PBMCs)

One hundred milliliters of whole blood was collected from each participant in heparinized tubes and PBMCs were isolated by density gradient centrifugation using Accuspin tubes (Sigma-Aldrich, St. Louis, MO) containing Histopaque®-1077, according to the manufacturer's protocol. Isolated PBMCs were resuspended at a concentration of 1×10^7 cells/mL in RPMI 1640 medium containing L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% dimethyl sulfoxide (Protide Pharmaceuticals, St. Paul, MN) and 20% fetal calf serum (Hyclone, Logan, UT), frozen overnight at −80°C, and transferred to liquid nitrogen for storage until use.

Growth of vaccinia virus

The NYCBOH strain of VACV was obtained from ATCC (Manassas, VA), and grown to make a master stock of virus used for the entire study as previously described (29). HeLa S3 cells were infected at a multiplicity of infection (MOI) of 0.05 with NYCBOH VACV for 3 d. Infected cells were collected, spun at 500 × g for 10 min at 4°C, and resuspended in 10 mM Tris-Cl (Sigma-Aldrich), pH 9.0. The cells were sonicated and centrifuged at 2000 × g for 10 min at 4°C. The supernatant, which contained the virus, was further purified by ultracentrifugation and titered according to established protocols (9,10). The titered VACV stock was diluted to 1×10^8 PFU/mL with 1 × Hanks' balanced salt solution containing 0.1% bovine serum albumin (Sigma-Aldrich).

The virus was inactivated by incubating VACV with psoralen (Sigma-Aldrich) at a concentration of 5 μg/mL for 10 min at room temperature. The VACV stock was then UV-irradiated for 60 sec at 365 nm in a UV crosslinker (Spectrolinker™; Spectronics Corporation, Westbury, NY). The inactivated viral stock was titered by plaque assay to ensure inactivation. The viral stock was divided into single-use aliquots and stored at −80°C.

Vaccinia-neutralizing antibody assay

We adapted a vaccinia-specific neutralization assay originally developed at the Food and Drug Administration (FDA) to measure neutralizing antibody titer (15,19). Briefly, heat-inactivated serum samples were serially diluted and then mixed with a known quantity of a recombinant β-galactosidase (β-gal) expressing VACV for 1 h. Serum samples were then incubated with HeLa cells overnight. Vaccinia immune globulin (VIG) was used as a positive control in each assay, while the negative control consisted of medium only. After an overnight incubation, the cells were lysed and β-gal activity levels were quantified using a colorimetric substrate as a surrogate marker of virus activity. Results are defined as the serum dilution that inhibits 50% of virus activity (ID_{50}). Each serum sample was tested in at least three separate experiments and the mean value was reported.

Vaccinia-secreted cytokine measurements

PBMC aliquots were thawed and rested as previously described (15,22,26). Briefly, the cells were thawed and resuspended in culture medium supplemented with 50 IU/mL of IL-2 (Proleukin®; Chiron, Emeryville, CA), then incubated overnight. The cells were then collected, washed, and resuspended in culture medium at a concentration of 2×10^6 cells/mL for use in the cytokine secretion assays.

PBMCs were plated in 96-well round bottom plates at a concentration of 2×10^5 per well. Experimental conditions for each subject consisted of a single PHA (5 μg/mL) well as a positive control, culture medium (unstimulated wells) measured in triplicate, and VACV (stimulated wells) measured in triplicate. Viral stimulation conditions were optimized as previously described (26), as follows: IFN-β, IL-2, IL-18: MOI = 5.0, 24 h; IL-12p40, IL-12p70, TNF-α, IL-1β: MOI = 0.5, 24 h; IFN-α, IFN-γ: MOI = 0.05, 4 d; IL-4, IL-10: MOI = 0.05, 7 d; IL-6: MOI = 5.0, 8 d.

Commercial ELISA-based kits for IL-1β, IL-2, IL-12p40, IL-12p70, TNF-α, and IFN-γ (all from BD Pharmingen, Franklin Lakes, NJ), IFN-α (PBL Biomedical Laboratories, Piscataway, NJ), IFN-β (PBL Biomedical Laboratories), and IL-18 (MBL International, Woburn, MA) were used to detect cytokines from culture supernatants according to the manufacturers' protocols. The optical density of each plate was measured at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA), and the concentration of each cytokine was

determined from manufacturer reference standards included on each plate.

IFN-γ Elispot assays

Total human IFN-γ and $CD8^+$ IFN-γ Elispot assays (R&D Systems, Minneapolis, MN) were performed on PBMC cultures (rested overnight in the presence of 50 IU/mL IL-2) as previously described (27,29). First, 2×10^5 PBMCs were plated in each well and stimulated with VACV at an MOI of 5.0. Three stimulated and three unstimulated replicates were used for each subject. PHA (5 μg/mL) was used as a positive control. Total IFN-γ plates were incubated for 24 h at 37°C in 5% CO_2, then developed according to the manufacturer's protocol. $CD8^+$ IFN-γ cells were incubated for 6 h at 37°C in 5% CO_2, then washed 3 times with PBS, then incubated for an additional 18 h at 37°C in 5% CO_2. The plates were then developed according to the manufacturer's protocol. All plates were scanned and analyzed on an ImmunoSpot® S4 Pro Analyzer (Cellular Technology Ltd., Cleveland, OH) using ImmunoSpot version 4.0 software (Cellular Technology Ltd.).

Statistical analysis

For purposes of this study, 15 immune response outcomes were examined: a measure of circulating VACV antibodies (reported as ID_{50} values); numbers of IFN-γ- and $CD8^+$ IFN-γ-secreting cells detected by Elispot (reported as number of spots per 2×10^5 cells); and 12 measures of VACV-specific *in vitro* cytokine secretion (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α, IFN-γ, IFN-α, IFN-β, and IL-18, each reported in units of pg/mL). Quantification of antibody titers resulted in three or more observations per subject. In contrast, assessments of cytokine secretion and Elispot values resulted in six recorded values for each of the outcomes of interest per individual: three without stimulation and three with VACV stimulation. For descriptive purposes, a single response measurement per individual was obtained for each outcome by using the median of the ID_{50} values for antibody titers, and by subtracting the median of the three unstimulated values from the median of the three stimulated values for cytokine secretion and Elispot value. Data were summarized across individuals using frequencies and percentages for categorical variables and medians and interquartile ranges (IQRs) for continuous variables. We assessed pair-wise correlations between immune response measures using Spearman correlation coefficients and corresponding 95% confidence intervals (CIs). All statistical tests were two-sided, and all analyses were carried out using the SAS software system (SAS Institute, Inc., Cary, NC).

Results

Clinical and demographic data

The median age of participants at enrollment was 24 y (IQR 22–27 y), and 74% of the study participants were males (n = 795) as previously reported (18). Racial data were derived from self-reported data collected during the consenting process: 53.1% of participants identified themselves as Caucasians (n = 572), 17.4% as African-Americans (n = 187), 6.9% as Asian (n = 74), and 22.6% as unknown or more than one race (n = 243). Additionally, 21.5% of study participants identified themselves as ethnic Hispanic or Latino (n = 231), and 4.2% as unknown ethnicity (n = 45). The median time from smallpox vaccination to enrollment in the study was 15.3 mo.

Vaccinia-specific immune responses

The vaccinia-specific humoral immune response, as previously reported, was measured by a high-throughput neutralizing antibody assay. ID_{50} values ranged from 15.71–1314.15, and the median ID_{50} value was 132.59 (IQR = 78.76–206.46) (18).

The concentrations of 12 secreted cytokines were detected in PBMC cultures stimulated with VACV by ELISA as outlined in Table 1. We predominantly detected IL-6 secretion, but we also detected moderate secretion of IFN-γ and TNF-α. We detected negligible levels of IL-12p70, IL-10, IFN-β, IL-18, and IL-4.

The number of IFN-γ- and $CD8^+$ IFN-γ-cytokine secreting cells were detected via Elispot. We detected a robust number of IFN-γ-secreting cells (median = 52 spot-forming units [SFU], IQR = 24–88 SFU), but a fewer number of IFN-γ-secreting $CD8^+$ T cells (median = 9.75 SFU, IQR = −2.00 to 26.50 SFU).

Correlation between neutralizing antibody titer and CMI measures

Using a Spearman test to determine the correlation between two data sets, we found a small positive correlation between secreted IL-2 and neutralizing antibody titer (correlation estimate 0.11; 95% CI 0.05,0.18; $p = 0.0009$), as well as a small correlation with secreted IFN-γ (correlation estimate 0.10; 95% CI 0.04,0.16; $p = 0.0007$). Additionally, we detected a statistically significant correlation between IFN-γ-secreting cells and neutralizing antibody titer (correlation estimate 0.08; 95% CI 0.02,0.11; $p = 0.006$).

Correlation between individual CMI measures

We also performed analyses to determine correlations between individual vaccinia-specific CMI responses. For purposes of this correlation analysis we excluded any secreted cytokines with a median detection level less than detectable levels as measured by the manufacturer's lowest

Table 1. Secreted Cytokine Levels of the Study Cohort

Cytokine	*Assays performed (n)*	*Median IQR[1] (pg/mL)*
IL-2	871	16.22 (3.79–36.99)
IL-4	1000	0.60 (−2.00–3.55)
IL-6	847	1071.67 (449.09–1944.52)
IL-10	1003	2.67 (−0.30–11.55)
IL-12p40	893	62.41 (29.48–122.38)
IL-12p70	890	3.02 (0.59–5.77)
IL-18	870	0.75 (−1.63–2.95)
IL-1β	921	50.80 (25.51–121.87)
IFN-α	1038	62.67 (16.85–132.79)
IFN-β	880	1.58 (−3.25–6.94)
IFN-γ	1038	289.51 (11.71–1,261.24)
TNFα	921	165.02 (91.05–319.25)

IQR, interquartile range.

Table 2. Statistically Significant Pair-Wise Correlation Between Vaccinia-Specific Cell-Mediated Immune (CMI) Responses

CMI measure	*Correlated CMI measure*	*Correlation estimate*	*Lower 95% confidence limit*	*Upper 95% confidence limit*	p *Value*
IL-1β	TNF-α	0.77	0.74	0.79	<0.0001
IL-6	TNF-α	0.61	0.57	0.65	<0.0001
IL-12p40	TNF-α	0.59	0.55	0.63	<0.0001
IL-12p40	IL-1β	0.56	0.51	0.60	<0.0001
IL-6	IFN-α	0.48	0.42	0.53	<0.0001
IL-6	IL-1β	0.46	0.41	0.51	<0.0001
IFN-γ Elispot	IFN-γ CD8$^+$ Elispot	0.45	0.40	0.50	<0.0001
IL-2	IFN-γ	0.40	0.34	0.45	<0.0001
IFN-α	TNF-α	0.40	0.35	0.46	<0.0001
IL-6	IL-12p40	0.32	0.25	0.38	<0.0001
IL-2	TNF-α	0.31	0.25	0.37	<0.0001
IL-2	IL-6	0.28	0.21	0.34	<0.0001
IL-1β	IFN-α	0.27	0.21	0.33	<0.0001
IL-12p40	IFN-γ	0.27	0.20	0.33	<0.0001
IL-2	IFN-α	0.26	0.19	0.32	<0.0001
IL-2	IFN-γ Elispot	0.25	0.18	0.31	<0.0001
IFN-γ	TNF-α	0.22	0.16	0.28	<0.0001
IL-2	IL-12p40	0.22	0.16	0.28	<0.0001
IL-1β	IFN-γ	0.22	0.16	0.28	<0.0001
IL-2	IL-1β	0.22	0.16	0.28	<0.0001
TNF-α	IFN-γ Elispot	0.21	0.15	0.27	<0.0001
IFN-γ	IFN-γ Elispot	0.21	0.15	0.26	<0.0001
IL-2	IFN-γ CD8$^+$ Elispot	0.21	0.14	0.27	<0.0001
IL-12p40	IFN-γ Elispot	0.19	0.12	0.25	<0.0001
IL-1b	IFN-γ Elispot	0.18	0.12	0.25	<0.0001
IL-6	IFN-γ CD8$^+$ Elispot	0.18	0.12	0.25	<0.0001
IL-12p40	IFN-α	0.16	0.10	0.23	<0.0001
IFN-α	IFN-γ Elispot	0.16	0.10	0.22	<0.0001
IFN-γ	IFN-γ CD8$^+$ Elispot	0.16	0.10	0.22	<0.0001
IFN-α	IFN-γ CD8$^+$ Elispot	0.15	0.09	0.21	<0.0001
IL-6	IFN-γ Elispot	0.15	0.08	0.21	<0.0001
TNF-α	IFN-γ CD8$^+$ Elispot	0.14	0.07	0.20	<0.0001
IL-6	IFN-γ	0.12	0.05	0.19	0.0004
IL-1β	IFN-γ CD8$^+$ Elispot	0.11	0.05	0.17	0.0008
IFN-α	IFN-γ	0.10	0.04	0.16	0.001

Spearman correlation coefficients with corresponding 95% confidence intervals. Only statistically significant correlations ($p \leq 0.001$) are presented.

cytokine reference standard dilution (IL-4, IL-10, IL-12p70, IL-18, and IFN-β). Despite using this stringent selection criterion, several individual CMI measures were significantly correlated with each other as demonstrated in Table 2.

Of note, we detected a very high positive correlation between the proinflammatory cytokines IL-1β and TNF-α, IL-6 and TNF-α, IL12p40 and TNF-α, and IL-12p40 and IL- 1β (correlation estimates 0.77, 0.61, 0.59, and 0.56, respectively). We detected a low positive relationship between secreted IFN-γ and the frequency of IFN-γ-secreting lymphocytes (correlation estimate 0.21), and a slight correlation between secreted IFN-γ and frequency of CD8$^+$ IFN-γ-secreting T cells (correlation estimate 0.16). No obvious pattern was present in the non-significant comparisons.

Discussion

This study focused on vaccinia-specific cellular immune responses following primary vaccination with Dryvax, as well as correlations between humoral and CMI responses, and correlations between individual vaccinia-specific CMI responses, which allowed us a macro level examination of immune response interactions following vaccination with VACV.

Many studies have characterized humoral immune responses following vaccination with VACV; however, relatively few studies have assessed CMI responses to VACV. A study comparing Dryvax, ACAM1000, and ACAM2000 vaccines detected robust and similar numbers of IFN-γ-secreting cells compared to those seen in our study (12). Additionally, three studies examining the kinetics of serum cytokines post-primary vaccination with VACV demonstrated that the majority of subjects had a $>50\%$ increase in serum IFN-γ and TNF-α levels 1 wk after primary vaccination with a VACV-containing vaccine (5,20,24).

Likewise, we also detected robust secretion of IFN-γ in PBMC culture supernatants after VACV stimulation (Table 1). However, we detected negligible levels of IL-4 and IL-10,

suggesting a Th-1-like cytokine pattern 15.3 mo (median) after primary vaccination with VACV. We also detected high concentrations of the proinflammatory and innate cytokines IL-6, TNF-α, IL-1β, and IFN-α, suggesting that the cytokine response to VACV was dominated by a proinflammatory cytokine response in concert with a Th-1-like cytokine secretion pattern. A strong cellular response was not unexpected in this cohort due to the inclusion criteria used for this study. Every participant in this study cohort exhibited a vesicular lesion or "take" at the vaccination site, indicating that the individual had an immune response to the vaccine (2,17).

Correlations between vaccine-induced humoral and CMI responses are ambiguous. For example, studies with hepatitis A and B vaccines have demonstrated that low- or non-responders, as determined by antibody levels, also have significantly reduced concentrations of antigen-specific IL-2, IFN-γ, and IL-10 compared to high-responders (13,14,30). Similarly, high IL-10 production has been inversely correlated with influenza antibody production following trivalent influenza vaccine vaccination (6). Contrary to these findings, our group and others have demonstrated minimal or no correlation between cellular immune responses and the antibody response to measles and rubella viruses following MMR vaccination (1,7,28).

In this study, we demonstrated that both secreted IL-2 and IFN-γ were significantly correlated with neutralizing antibody titers, although correlation estimates were modest (correlation coefficients ≥ 0.1). These findings were, however, highly significant ($p = 0.0009$ and 0.0007, respectively). IL-2 is known to promote differentiation of T-helper cells, which in turn stimulates and activates B cells, thus there is a possible biological explanation for the positive correlation seen between neutralizing antibody titer and IL-2 secretion. We also detected a positive correlation between secreted IFN-γ and neutralizing antibody titer, but we did not detect any correlation between neutralizing antibody and Th-2-like cytokines, which have been associated with B-cell maturation. However, this could be due to the low levels of Th-2-like cytokines. Although these findings were statistically significant, given the small correlation coefficient, further studies that precisely define the role of IL-2 and IFN-γ in response to VACV vaccination are necessary to determine precisely how these cytokines are related to the antibody response.

In terms of humoral immunity we detected a robust proinflammatory vaccinia-specific cytokine response, but we detected limited, non-significant correlations between all proinflammatory cytokines and neutralizing antibody titers. These data may imply that these two immune processes are under different regulatory control mechanisms, and that novel methods may be used to attenuate proinflammatory responses without diminishing the neutralizing antibody titer induced by VACV. Although not directly contrary to this idea, in a study examining vaccination with differing doses of Dryvax, Belshe *et al.* reported that subjects who had a fever (temperature $\geq 100°F$) 7–9 d after vaccination had a 1.4-fold greater neutralizing antibody titer following primary vaccination (3).

Additionally, we also found that several individual CMI responses correlated strongly with each other. Our strongest correlations were between the proinflammatory cytokines IL-1β, IL-6, and TNF-α (Table 2). We also observed strong correlations between these proinflammatory cytokines and IL-12p40, which is a well known proinflammatory cytokine stimulator. These data are expected, due to the fact that participants were vaccinated and PBMC cultures were stimulated with VACV, which has not been demonstrated to suppress proinflammatory responses (23). These strong correlations between certain inflammatory cytokines indicates that participants who secreted high levels of one proinflammatory cytokine also secreted high levels of all other proinflammatory cytokines, further demonstrating that VACV induces an overall robust proinflammatory host response.

Proinflammatory cytokines are needed to initiate an innate immune response to a pathogen, yet high concentrations of proinflammatory cytokines can induce undesirable AEs, including fever, headache, and inflammation (8). Our finding of a strong proinflammatory cytokine pattern suggests that some AEs associated with the Dryvax smallpox vaccine may be the result of high concentrations of vaccinia-induced proinflammatory cytokines. Novel methods that attenuate proinflammatory cytokine secretion to reduce proinflammatory cytokine levels to the minimal levels necessary to activate the innate immune system could result in new smallpox vaccines that are highly immunogenic, but have a reduced incidence of AEs.

Our study has multiple strengths, including a large sample population whose immune response was examined using a wide range of vaccinia-specific CMI response markers. On the other hand, our cohort had a limited sampling of females and a narrow age range, which could limit the generalizability of the results. Additionally, although we examined the vaccinia-specific cellular immune response, the cytokine responses were quantified in whole PBMC cultures, which contain a mixture of innate, T, and B cells. Isolating and stimulating individual cell subsets could potentially offer a clearer picture of host immune system activation by VACV. In the future we would like to measure cytokines secreted from different cell subsets, as well as increase the range of ages and balance the gender and racial distribution in the sample population to increase the generalizability of our data.

In summary, we detected a strong proinflammatory response in concert with a Th-1-like cytokine response pattern approximately 15 mo following smallpox vaccination. We detected a significant small correlation between secreted IL-2 and IFN-γ with vaccinia-specific neutralizing antibody titers. Further, we demonstrated strong correlations between multiple proinflammatory cytokines, indicating a strong host inflammatory response following vaccination with VACV. Interestingly, the inflammatory response was not correlated with neutralizing antibody titers, suggesting that it may be possible to further attenuate the licensed smallpox vaccine to minimize the inflammatory response without diminishing immunogenicity and seroprotective antibody responses.

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Author Disclosure Statements

No competing financial interests exist.

References

1. Allmendinger J, Paradies F, Kamprad M, *et al.*: Determination of rubella virus-specific cell-mediated immunity using IFN gamma-ELISpot. J Med Virol 2010;82:335–340.
2. Amanna IJ, Messaoudi I, and Slifka MK: Protective immunity following vaccination: how is it defined? Hum Vaccin 2008;4:316–319.
3. Belshe RB, Newman FK, Frey SE, *et al.*: Dose-dependent neutralizing-antibody responses to vaccinia. J Infect Dis 2004;189:493–497.
4. Belyakov IM, Isakov D, Zhu Q, *et al.*: Enhancement of CD8+ T cell immunity in the lung by CpG oligodeoxynucleotides increases protective efficacy of a modified vaccinia Ankara vaccine against lethal poxvirus infection even in a CD4-deficient host. J Immunol 2006;177:6336–6343.
5. Cohen JI, Hohman P, Fulton R, *et al.*: Kinetics of serum cytokines after primary or repeat vaccination with the smallpox vaccine. J Infect Dis 2010;201:1183–1191.
6. Corsini E, Vismara L, Lucchi L, *et al.*: High interleukin-10 production is associated with low antibody response to influenza vaccination in the elderly. J Leukoc Biol 2006;80:376–382.
7. Dhiman N, Ovsyannikova IG, Ryan JE, *et al.*: Correlations among measles virus-specific antibody, lymphoproliferation and Th1/Th2 cytokine responses following MMR-II vaccination. Clin and Exper Immunol 2005;142:498–504.
8. Dinarello CA: Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. J Endotoxin Res 2004;10:201–222.
9. Earl PL, Moss B, Wyatt LS, and Carroll MW: Generation of recombinant vaccinia viruses. Curr Protoc Mol Biol 2001; Chap 16:Unit 16.
10. Earl PL, Cooper N, Wyatt LS, Moss B, and Carroll MW: Preparation of cell cultures and vaccinia virus stocks. Curr Protoc Mol Biol 2001;Chap 16:Unit 16.
11. Empig C, Kenner JR, Perret-Gentil M, *et al.*: Highly attenuated smallpox vaccine protects rabbits and mice against pathogenic orthopoxvirus challenge. Vaccine 2006;24:3686–3694.
12. Frey SE, Newman FK, Kennedy JS, *et al.*: Comparison of the safety and immunogenicity of ACAM1000, ACAM2000 and Dryvax in healthy vaccinia-naive adults. Vaccine 2009;27: 1637–1644.
13. Garner-Spitzer E, Kundi M, Rendi-Wagner P, *et al.*: Correlation between humoral and cellular immune responses and the expression of the hepatitis A receptor HAVcr-1 on T cells after hepatitis A re-vaccination in high and low-responder vaccinees. Vaccine 2009;27:197–204.
14. Hayney MS, Buck JM, and Muller D: Production of interferon-gamma and interleukin-10 after inactivated hepatitis A immunization. Pharmacotherapy 2003;23:431–435.
15. Kennedy R, Pankratz VS, Swanson E, *et al.*: Statistical approach to estimate vaccinia- specific neutralizing antibody titers using a high throughput assay. Clin Vaccine Immunol 2009;16:1105–1112.
16. Kennedy RB, Ovsyannikova I, and Poland GA: Smallpox vaccines for biodefense. Vaccine 2009;27(Suppl 4):D73–D79.
17. Kennedy RB, Ovsyannikova IG, Jacobson RM, and Poland GA: The immunology of smallpox vaccines. Curr Opin Immunol 2009;21:314–320.
18. Kennedy RB, Ovsyannikova IG, Pankratz VS, *et al.*: Gender effects on humoral immune responses to smallpox vaccine. Vaccine 2009;27:3319–3323.
19. Manischewitz J, King LR, Bleckwenn NA, *et al.*: Development of a novel vaccinia-neutralization assay based on reporter-gene expression. J Infect Dis 2003;188:440–448.
20. McKinney BA, Reif DM, Rock MT, *et al.*: Cytokine expression patterns associated with systemic adverse events following smallpox immunization. J Infect Dis 2006;194:444–453.
21. Meseda CA, Garcia AD, Kumar A, *et al.*: Enhanced immunogenicity and protective effect conferred by vaccination with combinations of modified vaccinia virus Ankara and licensed smallpox vaccine Dryvax in a mouse model. Virology 2005;339:164–175.
22. Ovsyannikova IG, Jacobson RM, Ryan JE, *et al.*: HLA class II alleles and measles virus-specific cytokine immune response following two doses of measles vaccine. Immunogenetics 2005;56:798–807.
23. Ray CA, Black RA, Kronheim SR, *et al.*: Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. Cell 1992;69:597–604.
24. Rock MT, Yoder SM, Talbot TR, Edwards KM, and Crowe JE Jr: Adverse events after smallpox immunizations are associated with alterations in systemic cytokine levels. J Infect Dis 2004;189:1401–1410.
25. Rotz LD, Dotson DA, Damon IK, and Becher JA: Vaccinia (smallpox) vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2001. MMWR Recomm Rep 2001;50:1–25.
26. Ryan JE, Dhiman N, Ovsyannikova IG, *et al.*: Response surface methodology to determine optimal cytokine responses in human peripheral blood mononuclear cells after smallpox vaccination. J Immunol Methods 2009;341:97–105.
27. Ryan JE, Ovsyannikova IG, Dhiman N, *et al.*: Inter-operator variation in ELISPOT analysis of measles virus-specific IFN-gamma secreting T cells. Scand J Clin Lab Invest 2005;65: 681–690.
28. Tosh PK, Kennedy RB, Vierkant RA, Jacobson RM, and Poland GA: Correlation between rubella antibody levels and cytokine measures of cell-mediated immunity. Viral Immunol 2009;22:451–456.
29. Umlauf BJ, Pinsky NA, Ovsyannikova IG, and Poland GA. Detection of vaccinia virus-specific IFN-g and IL-10 secretion from human PBMC and CD8+ T cells by ELISPOT. Methods in Molecular Biology. 2011. (In press).
30. Velu V, Saravanan S, Nandakumar S, *et al.*: Relationship between T-lymphocyte cytokine levels and sero-response to hepatitis B vaccines. World J Gastroenterol 2008;14:3534–3540.
31. Vollmar J, Arndtz N, Eckl KM, *et al.*: Safety and immunogenicity of IMVAMUNE, a promising candidate as a third generation smallpox vaccine. Vaccine 2006;24:2065–2070.
32. Xu R, Johnson AJ, Liggitt D, and Bevan MJ: Cellular and humoral immunity against vaccinia virus infection of mice. J Immunol 2004;172:6265–6271.

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