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The immunology of smallpox vaccines

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In spite of the eradication of smallpox over 30 years ago; orthopox viruses such as smallpox and monkeypox remain serious public health threats both through the possibility of bioterrorism and the intentional release of smallpox and through natural outbreaks of emerging infectious diseases such as monkeypox. The eradication effort was largely made possible by the availability of an effective vaccine based on the immunologically cross-protective vaccinia virus. Although the concept of vaccination dates back to the late 1800s with Edward Jenner, it is only in the past decade that modern immunologic tools have been applied toward deciphering poxvirus immunity. Smallpox vaccines containing vaccinia virus elicit strong humoral and cellular immune responses that confer cross-protective immunity against variola virus for decades after immunization. Recent studies have focused on: establishing the longevity of poxvirus-specific immunity, defining key immune epitopes targeted by T and B cells, developing subunit-based vaccines, and developing genotypic and phenotypic immune response profiles that predict either vaccine response or adverse events following immunization.

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Introduction

Variola virus, the causative agent of smallpox, can be found throughout human history and probably developed alongside human civilization [1]. In 1798 Edward Jenner advanced the concept of using cowpox as a prophylactic agent against smallpox. Early practitioners used a wide

variety of pox viruses taken from or grown on cows, sheep, horses, goats, pigs, and buffaloes [1]. Vaccination quickly became widespread and by the 20th century almost all vaccines contained yet another orthopoxvirus: vaccinia virus (VACV) [1]. In spite of its indefinite origins vaccinia virus was the basis for extremely effective vaccines that, together with surveillance and monitoring led to the eradication of smallpox in 1980. Rare, but potentially life-threatening adverse events, led to the cessation of vaccine use among the general public, and recent vaccination programs have highlighted the risk of cardiovascular adverse events [2,3]. During the height of the eradication effort in the 1960s research efforts focused on humoral immunity, although the importance of cellular responses was predicted. In fact, there are two historical definitions of ‘protection’ that, while not mutually exclusive, do rely on humoral and cellular immune responses, respectively. These are (1) serum neutralizing antibody titer > 1:32 [4] and (2) the formation of a ‘take’ or vesicle at the vaccination site due to cellular immune responses to the local infection [1].

Terrorist activities in the early 21st century as well as imported outbreaks of monkeypox in the USA spurred renewed interest in biodefense countermeasures for these public health threats [5,6]. Faced with inadequate stocks of smallpox vaccine, an outdated vaccine production method, an increasing unvaccinated, and hence susceptible population, as well as a growing number of both immunosuppressed individuals and people with vaccine contraindications (heart conditions, cancer patients, organ transplant recipients, skin diseases such as eczema); research efforts focused on increasing our understanding of poxvirus immunity in order to develop safe and effective next-generation vaccines. In this review we will focus on the highlights of research regarding the mechanisms of disease protection elicited by smallpox vaccines.

Vaccines used during the eradication effort (Dryvax[®], APSV[®], Lancy-Vaxina[®], L-IVP[®]) are termed first-generation vaccines (last produced in the 1970s and early 1980s) and contained live vaccinia virus administered by puncturing the skin of the upper arm with a bifurcated needle (Table 1). Successful administration of the vaccine typically led to the development of a characteristic pustule at the vaccination site. Historically the development of this ‘take’ was considered evidence of protection [1]. Several recent studies have demonstrated that these live vaccines can be diluted 5–10-fold with no

Table 1

Smallpox vaccines and vaccine candidates

Vaccine	Virus strain	Usage	Details
Dryvax [®]	NYCBOH	Widespread use	Used in US during eradication. Highly effective. Lyophilized stock.
APSV [®]	NYCBOH	Widespread use	Used in US during eradication. Highly effective. Frozen liquid preparation.
Lancy-Vaxina	Lister	Widespread use	Used world-wide during eradication. Highly effective.
EM-63	NYCBOH	Widespread use	Used in Russia during eradication. Highly effective.
Temple of Heaven	Tian Tian	Widespread use	Used in China during eradication. Highly effective. Greater number of adverse events compared to NYCBOH and Lister vaccines.
ACAM1000	NYCBOH	Clinical trials	Tissue culture (MRC-5 cells). Equivalent immunogenicity to Dryvax [®] .
ACAM2000	NYCBOH	Clinical trials	Tissue culture (Vero cells). Equivalent immunogenicity to Dryvax [®] . FDA approved in 2008. Part of US National Stockpile.
CCSV	NYCBOH	Clinical trials	Tissue culture vaccine. Equivalent immunogenicity to Dryvax [®] .
Elstree-BN	Lister	Clinical trials	Tissue culture vaccine. Replacement for early Lister vaccines.
MVA	Ankara	Limited use	Lost 15% of genome through serial passage in chick embryo fibroblasts. Cannot replicate in human cells. Used in Germany with fewer adverse events. Immunogenicity may not be equal to replication-competent vaccines.
ACAM3000	Ankara	Clinical trials	Next-generation MVA-based vaccine.
IMVAMUNE	Ankara	Clinical trials	Next-generation MVA-based vaccine.
TBC-MVA	Ankara	Clinical trials	Next-generation MVA-based vaccine.
NYVAC	Copenhagen	Clinical trials	18 ORFs deleted. Improved safety profile. Not widely tested. Immunogenicity may not be equal to unattenuated live vaccines.
LC16m8	Lister	Limited use	Attenuated vaccine based on Lister strain. Used in Japan with good safety record. No efficacy data. Immunogenicity may not be equal to unattenuated live vaccines.
dVV-L	Lister	Clinical trials	Lister-based vaccine with UDG gene deleted to improve safety. No efficacy data. Immunogenicity may not be equal to unattenuated live vaccines.
Subunit	Various	R&D	DNA or protein-based subunit vaccines

Characteristics of common smallpox vaccines. Data for this table were compiled from several sources [1,5,9,50].

significant decreases in immunogenicity [7,8]. These vaccines induced robust humoral immunity characterized by high antibody titers capable of neutralizing and opsonizing viral particles, fixing complement, hemagglutination, as well as participating in antibody dependent cell cytotoxicity [1,13,14^{*}]. Similarly, these vaccines induced strong cellular responses capable of secreting effector cytokines such as IFN γ and lysing infected cells [1,13,25]. Together, these adaptive immune responses cleared the localized vaccinia infection at the immunization site and elicited long-lived memory responses capable of recognizing and clearing subsequent variola infections.

The second-generation vaccines, produced in the past 5–10 years, contain replication competent viruses produced in tissue culture and are designed as replacements for these early vaccines [5]. These replacement vaccines were commonly compared to Dryvax[®] and were designed to elicit similar levels of immunity [9]. Third generation vaccine formulations have focused on attenuated vaccinia strains (LC16m8, MVA, NYVAC, dVVL) with the hope that they have better safety profiles [9] (see Table 2 for adverse events associated with smallpox vaccines). Next-generation vaccine development is now focusing on a variety of subunit (protein and DNA-based) in order to create safer, yet still efficacious smallpox vaccines. This review will focus primarily on the immune responses generated by first-generation and second-generation vaccines.

We focus here on adaptive immune responses due to vaccination, but the crucial role that innate immunity plays in poxvirus pathogenesis should not be overlooked. Moulton *et al.* recently reported that mice lacking components of the complement system suffer from increased disease severity and mortality when challenged with ectromelia virus (ECTV) [10^{*}]. Poxviruses possess a number of crucial virulence factors that act as immunomodulatory proteins targeting key innate pathways such as interferons, chemokines, inflammatory cytokines, complement, and the toll-like receptor (TLR) family of pattern recognition receptors [11]. These innate responses, including chemokines, inflammatory cytokines, as well as pattern recognition receptors and their associated pathways (Figure 1) initiate the more robust adaptive immune responses, as is evidenced by a recent report showing that TLR signaling is crucial to the development of CD8 T cell memory following vaccinia infection [12^{*}].

B cell responses

Smallpox vaccine induces strong humoral responses that play a crucial role in protection against disease [13,14^{*}]. A prospective study by Mack *et al.*, found that neutralizing serum antibody titers > 1:32 were associated with protective immunity against smallpox disease [4]. Vaccinia Immune Globulin (VIG), prepared from serum of recent vaccinees, prevents infection of close contacts of smallpox victims and treats vaccine-related complications [15]. Defects in humoral immunity have severe consequences

Table 2**Adverse events associated with live smallpox vaccines**

Adverse event	Rate of occurrence
<i>Serious and/or life-threatening</i> (per million vaccinees)	
Death	1–2
Postvaccinal Encephalitis	3–9
Progressive Vaccinia	1–7
Eczema Vaccinatum	2–35
<i>Moderate</i> (per million vaccinees)	
Generalized vaccinia	40–200
Myopericarditis	100
Accidental inoculation	100–600
Bacterial Infection	Unknown
Non-infectious rashes	1–5%
<i>Mild (these are far more common, affecting 5–80% of vaccinees)</i>	
Itching	
Fever	
Lymphadenopathy	
Headache	
Nausea	
Pain at vaccination site	
Fatigue	
Muscle aches	
Chills	

Adverse reactions noted after smallpox vaccination. Rates of occurrence are based on the data combined from results during the eradication campaign as well as recent civilian and military vaccination campaigns [2,7,8].

during poxvirus infection. B cell deficient mice are unable to clear ectromelia infection in spite of detectable levels of anti-viral CD8⁺ T cell activity [16]. Similarly, a study infecting Rhesus macaques with monkeypox virus demonstrated that vaccinia-specific B cell responses are essential for protection [17]. Recent data demonstrate that vaccinia-specific antibody levels (both total IgG and neutralizing antibody) persist for decades and that vaccinia-specific memory B cells are functional, maintained for more than 50 years, and are able to mount a vigorous antibody response upon re-vaccination with Dryvax[®] [18]. Moreover, the Baltimore Longitudinal Study of

Aging, using 209 individuals who had been vaccinated as far back as 88 years prior, indicated that nearly 97% of individuals maintain both vaccinia-specific IgG and neutralizing antibodies at protection levels against smallpox [19].

Recently, protein microarrays have been used to characterize humoral immune response profiles to smallpox vaccines [20*,21*]. These studies have shown that antibody responses in humans display considerable inter-individual variation and are directed against multiple vaccinia virus proteins (see Table 3). The proteins targeted by humoral responses are predominantly viral structural, and membrane proteins, although responses to core proteins and proteins expressed only in infected cells have also been observed [22*]. Recent reports have shown that specific antibodies against both the intracellular mature (IM) and extracellular enveloped (EE) virions of vaccinia (and variola) are essential for optimal protective immunity induced by vaccination [23].

Newer studies of humoral immune response after smallpox vaccination seek to identify the repertoire of antigenic peptides recognized by vaccinia-specific B cells. For example, the viral B5R protein was found to be the main target of neutralizing antibodies in VIG [24]. Clearly defined humoral epitopes may inform development of new vaccine candidates, antibody-based therapeutics, and could provide further understanding regarding protection against smallpox.

T cell responses

Smallpox vaccine induces strong CD4⁺ and CD8⁺ T cell responses that peak at two to four weeks postimmunization and then contract to form a stable memory population of T cells that remain detectable for decades [13,25]. Interestingly, the CD8 T cell memory population appears to decline faster than memory CD4⁺ T cells [26]. It has long been noted that defects in cellular immunity lead to uncontrolled vaccinia infection, indicating that T cells

Table 3**Proteins targeted by T and B cells**

Epitope type ^a	# of Epitopes recognized ^b	Target protein characteristics ^c
B cell	9–15 ORFs (# of ORFs per subject not ascertained)	Predominantly proteins with late or early/late promoters. Almost exclusively membrane and core proteins
CD4 ⁺ T cell	>130 ORFs (~0–20 ORFs per subject)	Early, intermediate and late proteins, predominantly structural and membrane proteins as well as DNA replication enzymes.
CD8 ⁺ T cell	>190 ORFs epitope diversity within individuals is not well studied. Most subjects recognized more than 1 epitope.	Predominantly early proteins. Multiple functional categories (virulence factors, viral replication enzymes, transcription factors, structural proteins) are targeted by CTL.

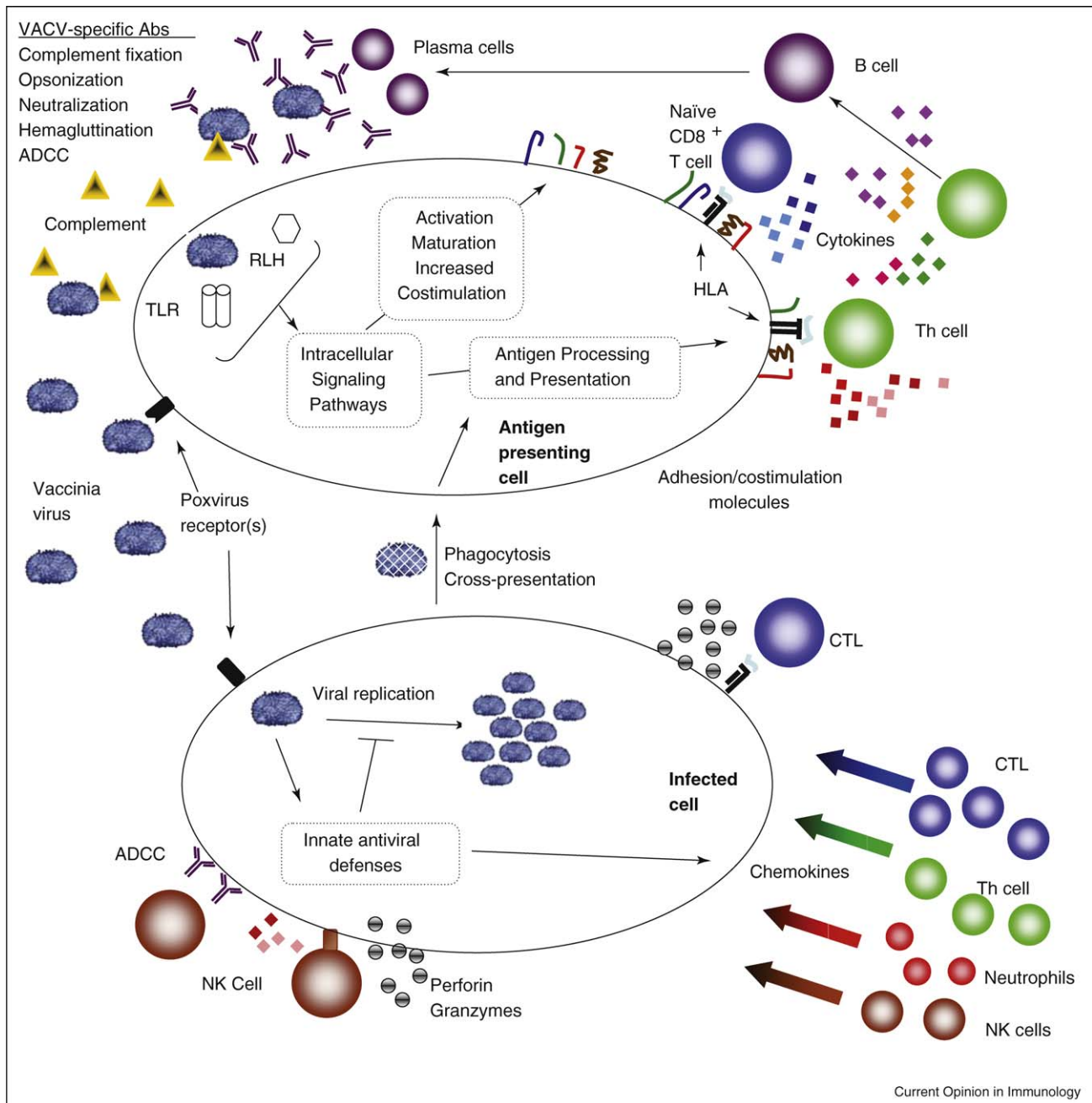
Immune epitopes from VACV.

^a Lymphocyte subset recognizing each group of epitopes.

^b Top number represents the total # of ORFs for which epitopes have been identified. The number(s) in parentheses indicate the extent or diversity of antigenic recognition on a per subject basis.

^c Details regarding the proteins targeted by each set of lymphocytes. Data collated from multiple reports identifying immune epitopes and from the Immune Epitope Database and Analysis Resource (<http://www.iedb.org>) [34,35,36,37*,39,40*].

Figure 1



Immune response pathways activated by smallpox vaccines. Immunization with the smallpox vaccines elicits a cascading network of integrated immune pathways. Non-specific innate responses activated by pattern recognition receptors serve to inhibit initial viral replication and to activate antigen presenting cells in order to properly initiate adaptive immunity. Innate inflammatory cytokines and chemokines then attract effector lymphocytes into infected tissues. T helper cells supply necessary cytokines (IL-4, IL-5) and costimulatory signals (CD40L) for the B cell maturation, replication and isotype switching. T cell help (IL-2, IFN γ) also promotes CTL activation, clonal expansion and effector function. VACV-specific T helper cells can also have direct lytic activity. B cells produce antibodies that agglutinate, opsonize, and neutralize viral particles, fix complement and allow for antibody dependent cell cytotoxicity (ADCC). Activated CD8 T cells lyse infected cells through perforin, granzymes, and through death receptors such as FasL. Cytokine secretion (IFN γ , TNF α) by T lymphocytes can also have direct antiviral activity. Together humoral and adaptive responses halt viral replication, lyse infected cells, and remove viral particles from the host. Virus-specific lymphocyte numbers then contract to a small, long-lived memory population capable of rapidly responding to subsequent infection with VACV and more. Electron micrograph of vaccinia virus adapted from the Centers for Disease Control and Prevention Public Health Image Library, image #2143.

play an important role in protection [27]. More recent data have shown that CD4⁺ and CD8⁺ T cells can prevent mortality in B cell deficient animals challenged with VACV [1,28]. Viral infection of CD4 deficient mice results in delayed viral clearance and increased mortality [29,30]. By contrast, animals lacking CD8 T cells are able to clear virus normally [31]. However, in the absence of humoral immunity, CD8⁺ T cells can provide partial protection [30], and immunization with a single HLA class I restricted epitope can provide varying degrees of disease protection [32]. The data illustrating how effective CD8 T cell responses are in the absence of humoral immunity greatly depend on the animal and the virus used. Studies using VACV as a challenge have shown that T cells are capable of delaying and in some cases clearing infection, while in models using species-specific pathogens (ECMV in mice or MXPV in non-human primates), CD8⁺ T cells are far less capable of viral clearance. The requirement for CD4⁺ T cells in protection is clear-cut as robust poxvirus-specific antibody responses fail to develop in animals lacking CD4⁺ T cells [17,30]. Similarly, CD4⁺ T cell help is essential for optimal CTL function and memory formation [33].

We have recently reviewed a large number of epitope mapping studies identifying well over 100 CD8⁺ T cell targets [34]. Additional efforts to pinpoint CD4⁺ T cell epitopes are underway as well [35–38].

In contrast to cellular responses to other pathogens, VACV-specific CD4⁺ and CD8⁺ T cells recognize a diverse array of viral proteins with no clear-cut patterns of immunodominance. CD8⁺ T cell epitopes are predominantly found in early, non-structural genes and transcription factors [39,40[•]]. Proteins encoded by early genes may be synthesized and presented more efficiently than those expressed late in the viral life cycle, and CTL specific for these epitopes are more likely to lyse infected cells before progeny virions are produced. By contrast, CD4⁺ T cell epitopes are concentrated in late, viral membrane and structural proteins as well as in enzymes involved in viral replication, and are capable of recognizing over 68% of viral proteins [37[•]]. Interestingly, new evidence suggests a close linkage between B cell and CD4⁺ T cell epitopes to vaccinia proteins [41^{••}]. Thus, viral proteins recognized by CD4⁺ T cells are also likely to be targeted by humoral responses, indicating that cognate T helper cell–B cell interactions may be required to generate robust VACV-specific antibody responses. Importantly, priming with CD4⁺ T cell epitopes can protect against lethal infection [41]. It is logical to assume that this protective effect may be more pronounced by the inclusion of nearby B cell epitopes, a conclusion that may account for the success of many subunit-based smallpox vaccines in animal studies [23,42–44].

Conclusions

Smallpox vaccines induce robust T and B cell responses that target a wide array of viral proteins and provide cross-protective immunity against important human pathogens such as variola and monkeypox. Recent advances in proteome-wide immune profiling and epitope identification have provided important information regarding poxvirus immunology. These types of studies that allow for deconstructing immune responses will probably be essential to the development of safer, next-generation vaccines and anti-viral therapies.

Another important avenue of research is in the understanding of the genetic factors influencing both vaccine response and adverse events. We have recently reported that gender is significantly associated with variations in neutralizing antibody titers developing after smallpox vaccination [45]. Stanley *et al.* have demonstrated that specific variations in the IL-1 and IL-18 genes are associated with the development of fever following smallpox vaccination [46]. Similarly, Reif *et al.* have identified single nucleotide polymorphisms in two genes (MTHFR, IRF1) that are associated with development of adverse reactions to Dryvax[®] [47]. McKinney *et al.* have identified patterns of serum cytokine expression, such as granulocyte colony-stimulating factor, stem cell factor, monokine induced by IFN- γ (CXCL9), intercellular adhesion molecule-1, eotaxin, and tissue inhibitor of metalloproteinases-2, after smallpox vaccination that may play a role in systemic adverse events [48]. These studies are significant in that a population could be screened for the presence of genetic or phenotypic profiles that predict serious adverse events before vaccination. One could similarly develop an immune profile that predicts the development of protective or ineffective vaccine responses, which would allow us to tailor more appropriate vaccination plans for these individuals [49]. The continuation of these studies will also improve our understanding of poxvirus pathogenesis, and may inform newer vaccine development.

Conflict of interest statement

Dr Poland has provided consultant advice to Acambis regarding smallpox vaccine development. The other authors do not have any conflicts of interest.

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