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Keeping the memory of Smallpox virus

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Abstract. Smallpox virus eradication was one of the greatest successes of the 20th century. Moreover, the quest to combat its use in biological warfare, has fueled efforts to understand residual immune memory and to develop new animal models by the scientific community. Although the literature is full of animal studies of vaccinia virus infection, continuing efforts have helped to increase our knowledge regarding humoral and cellular memory to non-persistent pathogens and to study factors that might influence further vaccination strategies in humans. In addition, the potent immunostimulatory action of poxvirus vectors has led to development and evaluation of new-generation vaccine candidates, which will be discussed in this review.

Keywords. T cells, humoral responses, vaccinia virus, vaccination, cellular memory.

Introduction

Smallpox is a viral infection that was eradicated from the world in 1978 [1]. This virulent disease, caused by variola virus, which is estimated to kill a third of those it infects, is known to have co-existed for thousands of years with human beings who struggled to find ways to battle smallpox. Variolation, developed in the 10th century in China and India, was a process that involved taking pus from the pocks of an individual suffering from smallpox and inoculating healthy people with it. Usually, they developed a mild case of smallpox, which thereafter conferred lifelong immunity. A major disadvantage of the practise was that variolated people could pass on severe smallpox to non-immunized individuals.

The real breakthrough in fighting the virus came in 1796 with the findings of Edward Jenner, who was quick to realize the enormous potential of vaccination [1]. It was only with advances in understanding vaccination that, in 1959, the World Health Organization (WHO) passed a resolution to undertake the global eradication of smallpox. Impressively, the eradication of smallpox was achieved with very little knowledge about the protective immunity raised after vaccination with vaccinia virus.

Studies on vaccinia-specific immune responses are divided into two eras: the period of circulation of smallpox and vaccination and, most recently, the absence of a human and/or animal reservoir for orthopoxviruses, at least in the Western countries. During the smallpox vaccination era when viruses were still circulating, studies suggested that protective immunity to smallpox declines 5–10 years after vaccinia inoculation. Accordingly, they recommended a vaccination schedule of priming at 1 year old and administering injections every 10 years [1]. The most widely investigated immune responses to vaccinia, i.e. neutralizing antibody responses, were considered to decline within the first 3–5 years after vaccination, and more recent small-scale studies confirmed this early decline of neutralizing antibodies [2, 3]. Since vaccination ended, people younger 30 years old are more prone to contract smallpox disease. In this new era of fear of biological warfare, and with the potential of modified vaccinia vector for future therapeutic and preventive vaccines, immunologists are keen to gain a better understanding of immune response. Recent studies have shown that T cell responses mediated by proliferating memory T cells or T lymphocyte precursors persist for up to 50 years after immunization [3, 4]. We will discuss the persistence of immune responses during these two eras in the presence and absence of circulating smallpox.

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Importance of immune response in smallpox disease

The ultimate goal of a vaccine is to develop long-lived immunological protection against pathogens through the development of a pool of memory cells and antibodies [5] (Fig. 1). Edward Jenner in the 18th century provided evidence that the initial encounter with pathogens may help protect against severe disease. We have now extensively increased our knowledge of poxvirus vaccination. Indeed, immunization by scarification using vaccinia-based vaccines generates potent induction of immune response. In humans and animal models, both humoral and cellular immunity types are crucial to combat poxviruses. Immunity to vaccinia virus involves both T and B cells, since defects in either compartment increase the risk of complication [6], although patients with T cell defects are far more susceptible to vaccinia viral infection than those with B cell defects [6]. However, this does not overshadow the importance of antibody responses that are involved in the inhibition of virus infection. Indeed, while B cell compartment does not seem to interfere with T cell compartment, the detriment to T cell compartment severely affects antibody response. Studies on complications of smallpox vaccination reveal that vaccinia necrosum, a life-threatening complication also known as progressive vaccinia, is mainly associated with T cell defects [7, 8]. It has also been shown that vaccinia immunoglobulin or adaptive transfer of T lymphocytes increase patient survival [6]. Further identification of protective immune responses has therefore become a fundamental issue in understanding immune response as well as in future vaccination strategies.

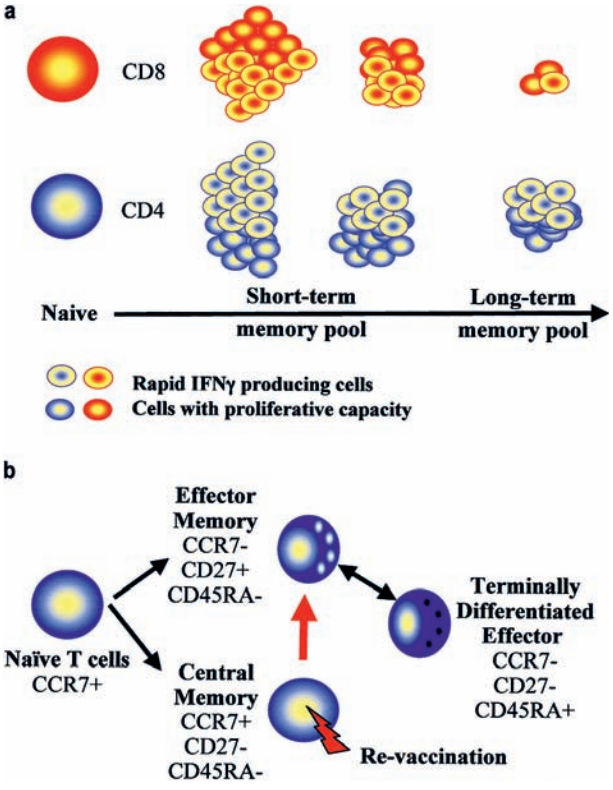

Figure 1. Differentiation of naïve T cell into effector memory cells. (*a*) Proposed model of maintenance of CD4 and CD8 rapid-IFNγ producing cells and proliferative memory cells specific to vaccinia virus. (*b*) Primary vaccination induces the differentiation of T cells into TEM and TCM. Re-vaccination reconstructs the pool of TEM pool long after primary vaccination (see also Ref 5).

Study of immune response during the era of circulating smallpox: before 1979

In the era of smallpox circulation prior to the 1980s, owing to technical considerations, studies were dominated by reports on the humoral immune response, while knowledge concerning T cell-mediated immunity was lacking. Neutralizing antibodies appeared at day 6 of illness during non-hemorrhagic smallpox, whereas antibody responses were lower and occurred later in patients suffering from hemorrhagic smallpox [9, 10]. Downie and colleagues demonstrated the persistence of vaccinia-specific antibodies after smallpox recovery: neutralizing antibodies persisted several years. On the other hand, hemagglutinin-inhibiting antibodies fell to a low level 5 years after infection, while complement fixation antibodies (which mediate destruction of virus-infected cells) rarely persisted after 1 year [11]. The same team showed that after vaccination, antibody production was delayed compared with smallpox infection, and no antibody was detected up to 10 days [9, 10]. During this period of smallpox circulation, neutralizing antibodies were demonstrated to persist up to 20 years in vaccinated individuals [9, 10].

Other studies indicated that vaccinated individuals retained immunity for a considerable period of time. Initial indications came from clinical observations based on examination of 1163 cases from the 1902–1903 smallpox outbreaks in Liverpool, England. These studies indicated that successful vaccination provided a high level of protection within 5 years of exposure but that immunity waned over time, although protection remained significant even after decades [12]. Another study of 680 cases conducted between 1950 and 1971 revealed mortality rates of 1.4% in individuals vaccinated 1–10 years before exposure, 7% in individuals vaccinated 11–20 years before exposure, 11% in individuals vaccinated more than 20 years before exposure and 52% among those who had never been vaccinated [13]. But the question remains that whether those vaccinated individuals maintained a long-lived immunological memory for vaccinia in the absence of circulating smallpox in the world.

Study of immune response decades after eradication of smallpox

B cell compartment

Even though T cell immunity is the focus of much literature, owing to notable technical advances in this field, neutralizing antibodies against vaccinia virus remain the principal route to controlling and eradicating infection. Recent studies have shown that more than 90% of long-term vaccinees retained vaccinia-specific immunoglobulin G (IgG) antibodies, and between 50 and 80% retained neutralizing antibodies [3, 14]. These vaccinia-specific antibody levels were remarkably stable between 1 and 75 years after vaccination [2, 3] (Fig. 2). Contrary to what was observed for vaccinia-specific T cell immunity, multiple vaccinations could improve both the fraction of responders and the level of the vaccinia-specific humoral response [15]. The percentage of individuals retaining long-term vaccinia-specific antibodies was lower among individuals who were vaccinated once, compared with individuals who received multiple vaccinations [15]. Moreover, antibody titers elicited after one vaccination were lower than those observed after two vaccinations [3]. Additional vaccinations did not further improve the antibody titer, and the authors concluded that 'booster vaccination may increase a previously suboptimal antibody response but is unlikely to induce prolonged synthesis of higher antibody numbers above a certain threshold' [3]. All vaccinees maintained some ability to neutralize intracellular mature virus (IMV), while a number of individuals lost the capacity to neutralize extracellular enveloped virus (EEV), particularly in the third decade after vaccination [16]. If neutralizing antibodies against both IMV and EEV are required for maximal protective immunity, decreased ability to neutralize EEV during time could have an impact on protection against smallpox. Interestingly, the ability to neutralize either of the virus forms was not altered by the number of vaccinations received [16]. In addition, Crotty et al. have recently shown that the neutralizing antibody titer correlated with anti-vaccinia antibodies in enzyme-linked immunosorbent assay (ELISA) [2] that tend to decline over years. But there is no correlation between vaccinia-specific T cells and antibody titers at early and late time points after vaccination, suggesting that humoral and cellular immunity types are independently regulated [3].

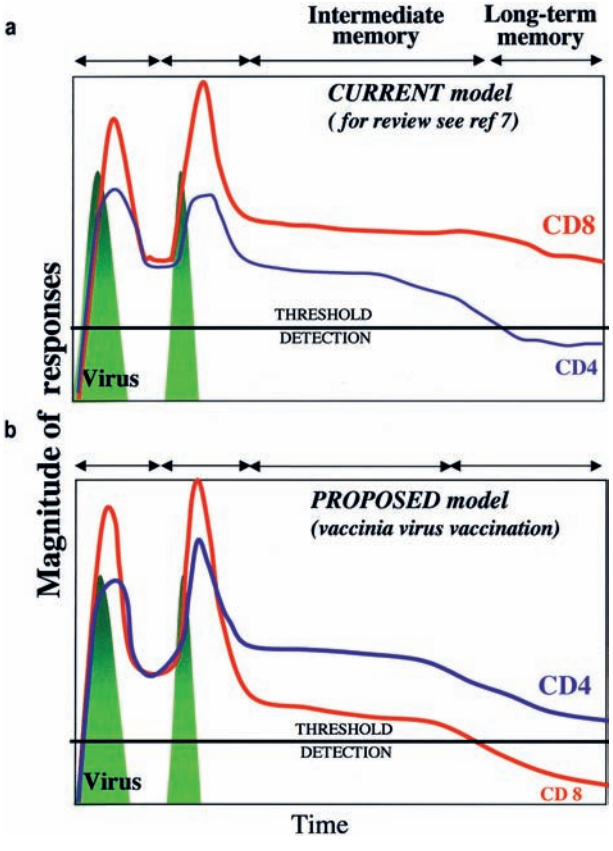

Figure 2. Comparative models of proposed amplification and maintenance of viral antigen-specific T lymphocytes. Current models showed that the magnitude of T cell responses is higher for CD8 memory cells than the CD4 memory cells (see for review Ref. 7). Study of vaccinia virus vaccination showed the reverse model where the magnitude of CD4 cells is higher than the one for CD8 memory cells (see also Ref. 5 and 28).

Recent studies using vaccines stocks of vaccinia virus have shown that the humoral immune response in humans and mice is directed against numerous antigens in the live Dryvax vaccine strain [17]. H3L, an intracellular mature virion envelope protein implicated in the attachment of vaccina virus to target cells, seems to be a major component of this antibody response, both in humans and mice [17, 18]. Purified human anti-H3L antibodies exhibited substantial vaccinia virus-neutralizing activity *in vitro* (50% plaque reduction neutralization test). Mice receiving H3L-neutralizing antiserum were protected from a lethal challenge with 3 LD50 (the amount causing death in 50% of the animals) of vaccinia virus strain WR (Western Reserve), demonstrating the *in vivo* protective effect of these antibodies [18].

T cell compartment

Earlier studies failed to detect vaccinia-specific cytotoxic T lymphocytes (CTLs) either in non-human primates or in humans [19–21], although poxvirus-specific CTLs were described in animal models [22, 23]. Perrin et al. advanced the hypothesis that non-T cytotoxic lymphocytes bearing Fc receptors and acting in the presence of specific antibodies were responsible for the vaccinia-virus cytotoxic activity observed in the PBMCs (peripheral blood mononuclear cells) of vaccinia-immune healthy humans [19]. Due to technical progresses, subsequent studies succeeded in detecting vaccinia-specific CTLs among CD4+ then CD8+ T cells [24, 25]. As our knowledge of memory and effector T cell response has con-

tinually increased over the past years, vaccinia virus-immune response particularly in regard to T cell immunity has been more recently widely evaluated by many groups [3, 4, 26–28]. Studies have performed classical laboratory assays to detect vaccinia-specific effector/memory T cells [by using IFNγ (interferon γ) ELISpot assay and intracellular cytokine staining (ICS)] and central memory T cells (lymphoproliferation assay).

The long-term persistence of cytotoxic T cell memory 35–50 years after immunization was evidenced for the first time in 1996 [29]. More recent study by Hammarlund et al. showed that more than 90% of volunteers vaccinated 25–77 years ago still maintained humoral and cellular responses against vaccinia [3]. In addition, we have shown that long-term proliferative memory response persisted in more than 70% vaccinated individuals 25 years after the end of the vaccination era [4]. However, the persistence of memory response depends on several types of memory cells that can be distinguished as follows: (i) rapid effector immune responses as measured by short-term production of IFNγ by T cells (T effector memory, TEM) and (ii) memory T cells with proliferative capacity (T central memory, TCM) [4] (Fig. 1). TCM cells capable of producing interleukin (IL)-2 and of proliferation, and are involved in clonal expansion of vaccinia-specific T cells, as opposed to the TEM cells which have lower proliferative and survival abilities and are involved in antigen-specific effector functions [4]. The phenotype of vaccinia-specific TEM has been defined as CD45RA-CD27+CCR7-CD11a^{Hi}, whereas the phenotype of proliferative vaccinia-specific TCM has remained technically difficult to determine [4].

In 79 individuals among the French population, only 20% of the vaccinees displayed both immediate IFNγ-producing TEM and TCM responses, as against the 72.5% who showed proliferative responses [4]. These results are in accordance with the study conducted by Kennedy et al. on 20 vaccinees, revealing that 20% exhibited a TEM response and 42% a TCM response [27]. Thus, TCM cells seem to have a greater capacity to persist *in vivo* and should help mediate a stronger protective immunity than TEM cells, although the evidence is not convincing in the absence of circulating smallpox.

Although the question of the quantity of T cell memory has been resolved, the quality of persisting T cells against vaccinia virus remains under investigation, and probably depends on the sensitivity of available techniques. The issue is important in that the CD4 Th1 cells were shown to be the principal component of the long-term cellular memory to vaccinia [4, 26] (Fig. 2), because CD8 effector and memory cells play a crucial role in viral infections [5]. About 50% of long-term vaccinees specifically lose vaccinia-specific CD8 T cell responses [3, 26]. We will see below that CD8 memory predominates at earlier stages after antigen exposure, suggesting that in humans, long-term CD8 memory cells for vaccinia are less stable than their CD4 counterparts. However, these data conflict with the conventional wisdom that CD8 T cells predominate in the memory of live viruses (for review see [5]). Explanations of this proposed CD8 predominance rely on the higher potential for *in vivo* expansion and better survival potential of antigen-specific CD8 cells, compared with CD4 T cells, which did present lasting memory after early immunization. Differences in contraction phases of vaccinia-specific CD4 and CD8 T cells were shown in humans, explaining the manifestation of higher frequencies of vaccinia-specific memory CD4 T cells as compared with CD8 T cells in the long term [26]. Consistent with the findings, one can hypothesize that the maintenance of CD4 helper cells is more important in the persistence of B cell memory and antibody production in the absence of circulating antigens.

Factors influencing the persistence of T cell memory to vaccinia virus

T cell-mediated immunological memory, widely studied in mouse viral infection models, requires a clonal expansion of naive T cells during the first virus encounter, followed by the contraction phase with virus clearance, and consequently by the persistence of a stable number of antigen-specific memory T cells that rapidly proliferate with antigen re-exposure. The initial priming dose of vaccine determines the frequency and the final numbers of persisting antigen-specific CTLs [5]; however, the factors necessary for the persistence of memory T cells require further investigation. It is unclear whether priming or periodic re-exposure to antigen is needed to maintain high frequencies of memory T cells.

The initial size of the CD8 effector compartment correlates with the magnitude of the long-term memory response (Fig. 1). Ongoing studies have proposed various models and suggested that either TEM and TCM are independent subpopulations or TEM is at the end-stage of TCM differentiation. Another key point is localization of the TCM and TEM in lymphoid organs or in circulating cells. Indeed, most studies are done with peripheral blood cells and thus can visualize only circulating memory cells.

Many factors determine the frequency of CD4+ and CD8+ effector T cells: (i) antigen exposure and intensity of the initial T cell burst [5]; (ii) the kinetics of CD8 T cell proliferation, which differs from that of CD4 (naive CD8 T cells require less antigen exposure to develop their proliferative program but also divide faster than CD4) [30]; (iii) survival of memory T cells is regulated by complex homeostatic mechanisms [5]; and (iv) cross-reactivity with other pathogens might also determine memory cell survival [31]. Mechanisms of transition of effector to resting memory T cells are not fully understood and

seem to imply IL-7 in the generation of CD8 memory T cells [32]. Further studies demonstrated that CD4 T cells are implicated in the generation of competent memory CD8 T cells [33–35].

Survival of memory T cells is regulated by complex homeostatic mechanisms depending on prior antigen exposure and implicating cytokines such as IL-15 and regulation of apoptosis [36]. After differentiation into memory cells, neither CD4 nor CD8 T cells require further stimulation with specific or cross-reactive antigen for their maintenance [37, 38]. However, in a mouse model after a peak during acute viral infection, CD4 memory T cells decreased slowly in the absence of antigen, whereas CD8+ T cell memory was stably maintained for life [39]. Animal studies found comparatively stable CD4+ memory for both readily cleared influenza virus and LCMV (lymphocytic choriomeningitis virus) [40, 41] and persistent γ-herpes virus [42]. These data suggest that exposure to antigen after acute infection is not necessary to maintain memory.

We and others demonstrated a decrease of vaccinia-specific immune effector cells in vaccinated individuals 45 years after immunization [3, 4]. This decrease does not simply reflect aging, because tuberculin responses, generated by the BCG (Bacille bilié de Calmette-Guérin) vaccine during childhood, had been maintained in the same individuals [B. Combadière and B. Autran, unpublished observations]. However, memory to these two vaccines might differ because BCG-vaccinated individuals are exposed to cross-reactive mycobacteria, while smallpox-vaccinated individuals are not. Some cross-reactivity has been reported with other viruses (*Molluscum contagiosum*, LCMV) that might participate, to some extent, in the maintenance of vaccinia-specific memory.

When analyzing the factors influencing persistence of memory to smallpox/vaccinia, we found that delay of first antigen encounter had a major impact on the maintenance of immune response. Anti-vaccinia proliferative T cell responses were maintained over the years after antigen exposure [3, 4], but vaccinia-specific effector/memory T cells vanished 45 years after the first vaccinia inoculations. Neither the time since the last immunization nor the number of immunizations influence the maintenance of vaccinia-specific T cells [4]. The observation that periodic re-exposures to the vaccine do not increase the size of the residual effector/memory pool when measured decades after the last immunization suggests that priming exerts a stronger influence than subsequent exposure on its long-term persistence. However, in the Taiwanese population, Hsieh and colleagues found that T cell reactivity to vaccinia waned within 20–30 years after T cell priming [43]. The difference observed in the maintenance of vaccinia-specific CD4+ and CD8+ T cells could be due to the fact that CD4 and CD8 memory T cells might differ by their antigen dependence. According to an emerging consensus from murine models, long-term CD8 memory does not require antigen persistence [44–46]. Virus-specific CD4 T cell memory resulting in single exposure to readily eliminated Sendai virus can also be maintained for more than 2 years [47]. Both variola and vaccinia viruses are thought to be completely cleared from the organism after acute infection or inoculation, suggesting that vaccinia-specific memory CD4 T cells persist even better than CD8 T cells despite the absence of both viruses for the last 25 years. The amount of antigen received during the first immunization might nonetheless have determined the formation of the vaccinia-specific memory pool [5]. While we assumed that all vaccinated individuals had received similar vaccinia doses, the actual number of virus particles penetrating the skin was known to vary widely among vaccinees, and diluting the vaccine reduced the rate of successful vaccination.

Results regarding long-term immunological memory in humans led us to propose a new model of intermediate-term memory (less than 45 years after antigen priming) where rapid effector/memory responses are essentially composed of CD4 Th1 cells, and a long-term memory (more than 45 years after antigen priming) which depends mainly on the presence of CD4 T cells, even though CD8 memory T cells remain expandable after *in vitro* antigen exposure (Figs. 1, 2).

Epitope recognition in vaccinia virus vaccination

The use of viruses in vaccination has aroused interest in the study of immunodominance to large viruses. Immunodominance is defined as the phenomenon whereby only a small fraction of all of the protein determinants elicit an immune response. From the predicted 258 open-reading frames obtained in an expression library, five determinants of vaccinia virus (VACV, Dryvax vaccine) were identified [48, 49]. These five determinants accounted for half of the CTL responses observed in mice models. The hierarchy of recognition is highly affected by the route of VACV administration. Two out of five determinants failed to be recognized after MVA (modified virus Ankara) administration, a candidate for smallpox vaccination [48]. However, > 70% of the identified epitopes were highly conserved in variola virus and MVA. In addition, 48 epitopes restricted to various HLA (human leucocyte antigen) types described by Tscharke et al. are available for further studies of vaccinia virus-specific T cell responses [48]. Immunogenic residues were heavily biased toward early and virulence factor-related virus products. Among them, class-I MHC peptides have been reported for B8R, D1R, D5R, C10L, C19L, C7L, F12 and O1L [48, 49]. It must to be noted that most CTL responses are observed during primary vaccination, whereas residual long-term memory responses are mostly

associated with CD4 compartments. Efforts need to be made for further characterization of major histocompatibility complex (MHC)-class II epitopes. However, one study provided some evidence that vaccinia virus infection can disrupt MHC-class II presentation of antigens by antigen-presenting cells *in vitro* [50]. This mechanism could be part of the multiple mechanism of viral escape.

Recent advances in study of short-term immune response after primary vaccination and re-vaccination

Because of the risk of a bioterrorist attack with smallpox virus, there is an urgent need to determine optimized conditions of use of vaccine after prolonged storage (indeed, vaccinia vaccine was not manufactured over 20 years ago) and to obtain additional vaccine doses out of the present stock. To counter the availability of limited vaccine doses in the case of mass vaccination, immune response was also evaluated with a low dose of vaccine. Accordingly, clinical trials and animal models addressed the questions of safety, immunogenicity, and doses of old and recent vaccinia virus strains.

Primary vaccination with licensed smallpox vaccine (Dryvax) administered by the standard scarification method induced strong humoral and T cell responses (IFNγ-producing and proliferative responses) [28, 51, 52]. Both the vaccinia-specific T cells, CD4 and CD8, appeared between 1 and 2 weeks following primary vaccination with Dryvax and peaked at week 2 [26, 27]. Amara et al. showed that the magnitude of CD8 response was 2- to 4-fold higher than that of CD4 (mean 1.37% versus 0.33%) [26]. This primary response was biased toward type 1 cytokines such as interferon γ (IFNγ), tumor necrosis factor α (TNFα) and IL-2. Interestingly, as mentioned above, vaccinia-specific CD4 and CD8 T cell contraction phases were different: the vaccinia-specific CD8 response contracted 7-fold, while the vaccinia-specific CD4 response contracted 2-fold [26], leading to the maintenance of a dominant CD4 T cell response. The induction of both vaccinia-specific antibody responses and T cell responses (effector IFNγ-producing and proliferative responses) in vaccinia naïve subjects correlates with the presence of vesicles [51–53]. In addition, a small-scale study reported that after re-vaccination, T cell responses years after the last immunization were comparable in subjects with and without a take, defined by the presence of vesicles [54]. T cell immune responses after a re-vaccination were comparable to the primary response described above: by 2 weeks after the boost, robust CD4 and CD8 T cell responses were detectable (mean 0.22% and 0.34%, respectively) [26]. Then, by 12 weeks, CD4 contracted less than 2-fold, while CD8 contracted 5.5-fold. However, Kennedy found an earlier development of vaccinia-specific T cell response by day 7 in 63% of re-vaccinees [27]. We have demonstrated that vaccination of immunized individuals reconstituted the pool of both effector/memory CD4 and CD8 cells; however, CD4 response remained at higher intensity [4] (Fig. 2). Ennis and colleagues reported that vaccinia re-immunization was associated with a transient decrease of *in vitro* proliferative responses of PBMC to mitogens and recall antigens, as was previously observed during other viral infections [55].

After Dryvax vaccination, >94% of subjects, both vaccinia naïve and those previously vaccinated, displayed significant antibody response [52, 56]. As expected, the antibody responses appeared more rapidly in re-vaccinees (by day 7) compared with primary vaccinees (by day 14) and peaked at day 28 in the two groups [27]. Immune responses, however, were higher in pre-immunized than in the naïve individuals [57]. Four weeks after primary vaccination, 1.5% of circulating IgG+ memory B cells presented as vaccinia-specific. Such vaccinia-specific memory B cells initially exhibited exponential decay curve and then reached a plateau and persisted up to 60 years post vaccination [2]. Moreover, anti-vaccinia antibody (neutralizing and total) titers decreased and reached a plateau 1 year after vaccination [2]. Some individuals without smallpox vaccination history displayed vaccinia-specific antibodies [53, 58], probably due to the exposure to other circulating orthopoxviruses generating antibodies that cross-react *in vitro* [59].

In an attempt to increase the number of individuals who could be vaccinated in case of bioterrorist attack, dose-response studies were performed using diluted and undiluted smallpox vaccine both in vaccinated and unvaccinated subjects [51, 56, 60]. A first trial reported that cytotoxic T cell responses were lower, while neutralizing antibody responses were higher in vaccinia-naïve subjects receiving 1:100 than in those receiving undiluted Dryvax vaccine [51, 60]. In contrast, diluted Lister strain was shown to elicit humoral and cellular immune responses in the same range as those elicited with undiluted Lister, if viral titers were above 10^8 and $10^{7.5}$ pfu (plate-forming unit)/ml after dilution in vaccinia-naïve or re-vaccinated subjects, respectively [56]. This discrepancy could be explained by the fact that Frey and colleagues used a vaccinia strain at $10^{7.5}$ pfu/ml, leading to $10^{6.3}$ pfu/ml after 1/32 dilution. These results underscore the importance of the viral titer over the dilution ratio in evaluating diluted vaccines.

To enhance the safety of vaccines and lessen the risk of transmission with infectious vaccinia present in the local lesion after scarification, vaccines administered intradermally or intramuscularly were evaluated for clinical and immunological responses [53]. It was observed that intradermal administration did not systematically prevent formation of a pox lesion. Furthermore, intradermal or intramuscular administration of vaccinia vaccine without the concomitant development of a cutaneous pox lesion

was less reactogenic than scarification and induced lower cellular and humoral immune responses, suggesting that the development of a cutaneous pox lesion is necessary to mount robust vaccinia-specific immune response [53].

In this context of research of new vaccinia/smallpox vaccines, it has to be noted that the immunological correlates of protection against smallpox in human have not yet been fully defined. As discussed by Hammarlund, the fact that smallpox vaccination protects at least 90–95% of vaccinees against lethal smallpox infection [13] and that vaccinia-CD8 T cells persist only in 50% of vaccinated subjects could indicate that antibodies and CD4 T cells constitute the main components of long-term immunity against smallpox. Studies addressing this question in humans and in animal models show contradictory results. One study reported that previous smallpox vaccination did not influence illness severity or inpatient hospitalization [61]. Edghill-Smith et al. showed that antibodies are essential and sufficient to protect rhesus macaque from a lethal monkeypox infection [62]. Three studies in mouse models reported discordant conclusions: the first found that both CD4 and B cells are essential to protect against disease; a second revealed that CD4 T cells alone show the desired effect; and the third showed that antibodies alone protect against disease [63–65]. In addition, smallpox vaccination seems to protect against monkeypox disease, a smallpox-like disease caused by a zoonotic orthopoxvirus endemic in Congo and West Africa [66, 67].

New generation of vaccinia-virus vaccines

Attenuated vaccinia strains

Concerns about the safety of live replicative vaccinia strains have raised the utility of novel approaches in smallpox vaccination as early as the 1930s by evaluating the method of administration, the use of less-virulent replication-competent vaccinia and the use of attenuated replication-defective vaccinia viruses such as MVA (Modified Virus Ankara) and NYVAC (for review see [68]). MVA was obtained after more than 500 serial passages in chicken embryo fibroblasts, whereas NYVAC was generated from vaccinia Copenhagen strain by the specific deletion of 18 open-reading frames. These two attenuated vaccinia strains are unable to efficiently replicate in human cells. Apart from an abortive replication cycle, MVA also presents some immune advantage compared with vaccinia virus strain. MVA does not express soluble homolog receptors for IFNγ, IFNα–β or CC-type chemokines, although it enhances cellular genes implicated in the immune memory response, such as IL-6, IL-7 and IL-15 [69]. Both MVA and NYVAC were shown to be safe in immunosuppressed macaques [70, 71]. NYVAC was able to induce neutralizing antibodies to vaccinia, correlating with complete resolution of the skin lesions [70], even in macaques with severe CD4+ T cell depletion. This finding has a direct impact on the vaccination strategy in immunosuppressed individuals, for example in human immunodeficiency virus (HIV)-infected and transplanted patients.

A third-generation vaccine derived from MVA via additional passages in serum-free chicken embryo fibroblasts, named MVA-BN, was evaluated in a phase I clinical study in both naïve and re-vaccinated healthy controls [58]. MVA-BN was shown to be safe and elicited humoral response in a dose-dependent effect. Neutralizing antibodies peaked at day 28 in re-vaccinees (89% responders) and at day 42 in naïve subjects (80% responders) [58]. To our knowledge, no study evaluated immunogenicity between attenuated vaccinia NYVAC/MVA versus VV in humans, but Vollmar and colleagues observed that immune responses elicited with MVA-BN were in a range similar to those elicited by the conventional vaccine Dryvax [52, 58].

Evaluating the efficacy of new-generation vaccines is complex, since eradication of smallpox precludes a clinical efficacy trial, and the correlates of protection against smallpox are unknown. In this context, MVA and NYVAC were shown to be as effective as Dryvax in eliciting immune response and in protecting against vaccinia or cowpox challenge in animal models [63, 72–74]. MVA also protected macaques against respiratory and intravenous monkeypox challenge [71, 75] and elicited humoral and T cell responses in the same range as Dryvax [75].

Vaccination with recombinant vaccinia viruses

Recombinant-attenuated vaccinia vectors (r-VVs) expressing viral genes might be one of the most promising strategies for combating viral infection [76] (for review see [77]). The fight against many infectious diseases (HIV, tuberculosis, malaria etc.) as well as the search for new vaccines has led to major progress in the development of MVA recombinant vectors [78–81].

Poxvirus vectors and, more particularly, vaccinia viruses have been proposed as vaccine candidates for decades because the flexibility of their genome allows deletion of large parts of it and insertion of foreign genes. The ability of these vectors to replicate expression of a large number of recombinant genes, together with their ability to stimulate both innate and adaptive immune responses provides an opportunity to induce an efficient adaptive immune response against recombinant proteins. These vectors are also easy to produce [82–84].

In this context, information about the persistence of long-term vaccinia-specific immunity is crucial, because such immunity could impair the efficacy of immune response against foreign antigen. Indeed, during repeated immunizations, a strong response against viral vector was associated with a lower response against recombinant antigen

[3, 4]. This is particularly relevant in the case of vaccinia because a large human population received vaccination. Pre-existing humoral immunity against vaccinia virus was shown to negatively influence both the titer and the duration of the antibody response induced by a second recombinant-vaccinia vector and to reduce protective immunity against the pathogen [85, 86]. The impact of pre-existing cellular immunity has been studied to a lesser extent but was shown to contribute to immunity against adenovirus vector and to decrease the immunogenicity of recombinant adenovirus [87]. Studies in mouse models showed that although prior immunity to an MVA vector causes a significant decrease in cellular and humoral immune response to foreign antigen, this immune response was still detectable and higher than that observed when mice were pre-immunized with vaccinia WR [88]. In mouse models, MVA recombinants were shown to induce a strong immune response against the recombinant antigen and a poor immune response against the vector itself [88, 89]. Vaccinations with r-VVs elicited responses against both the vector and the foreign antigen. Harrington et al. showed that T CD8 immunity against the vector was 20- to 30-fold higher than immunity to foreign antigen in a mouse model [90]. Interestingly, the kinetics of immunity to foreign antigen seem to parallel the kinetics to vector [90]. Thus, the use of r-MVA in preclinical and clinical research also requires the evaluation of immune response to MVA after immunization, particularly in individuals who have been vaccinated against vaccinia virus where the immunity to the vector can be very high.

Clinical trials are currently underway in humans, particularly in the fields of HIV, tuberculosis and malaria vaccine research [78–81]. Previous studies showed an improvement in cellular immune response and protection against pathogens such as simian immunodeficiency virus (SIV) and *Plasmodium* species following prime/boost regimens associating DNA and MVA in both animals and humans [91–94]. Experiments in mouse models using DNA and r-MVA prime boost regimens are promising and showed protection against *Plasmodium* challenge [92]. However, different malaria vectors need to be used in a specific order for an optimal *ex vivo* IFNγresponse [95]. NYVAC-Pf7, a highly attenuated vaccinia virus with seven *P. falciparum* genes, was safe and well tolerated but variably immunogenic: antibody responses were poor, while cellular immune responses were detected in >90% of the volunteers in phase I/II trials [96]. Combination with other poxviruses such as fowlpox was recently proposed. Attenuated fowlpox virus and MVA recombinant for a malaria pre-erythrocytic antigen used in prime-boost regimens with DNA were safe and immunogenic in humans and generate promising protective efficacy against pathogens [97, 98]. Moreover, a prime-boost strategy using DNA and r-MVA elicited a high level of anti-malaria T cells and partial protection against challenge in humans [94].

In the context of HIV research, MVA and NYVAC were shown to induce potent HIV-specific immune response both in mice [99, 100] and macaques [93, 101, 102]. It seems that fowlpox and vaccinia viruses as well as MVA were comparable in their ability to boost DNA primed CTL in an SIV model in monkeys [103]. Clinical trials evaluating recombinant MVA in humans showed that r-MVA Gag HIV clade A and r-MVA Nef were safe and immunogenic in healthy controls [104, 105] or in HIV-infected patients [106].

In addition, strategies have been proposed to improve the immunogenicity of recombinant vaccinia vectors. The immune responses elicited by recombinant MVA can be improved with the co-delivery of IFNγ or IL-12 by priming with DNA vector co-expressing antigen and IFNγ [100] or by using r-MVA- IFNγ or r-MVA- IL-12 [107]. However, as a result of fear of bioterrorism, the effect of modified IL-4 expressing VV was tested in mouse models. McCurdy et al. showed that vaccination with MVA protects even when mice are challenged with recombinant vaccinia virus expressing murine IL-4, which triggered a greater Th2 response [73].

In addition to the fear of biological warfare and the need to determine residual immune memory to smallpox, over the past decades, immunologists have made strides in understanding the mechanism of immune response required for efficient vaccines.

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