Ian J. Amanna
Mark K. Slifka
Shane Crotty

Immunity and immunological memory following smallpox vaccination

Authors' address
Ian J. Amanna[1], *Mark K. Slifka*[1], *Shane Crotty*[2]
[1]OHSU Vaccine and Gene Therapy Institute, Beaverton, OR, USA.
[2]Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, San Diego, CA, USA.

Correspondence to:
Shane Crotty
Division of Vaccine Discovery
La Jolla Institute for Allergy and Immunology
10355 Science Center Drive
San Diego, CA 92121
USA
Tel.: +1 858 678 4559
Fax: +1 858 558 3525
E-mail: shane@liai.org

Acknowledgements
This work was supported in part by NIH/NIAID contract N01 AI40029, and grants AI063675, RR00334 to M.K.S and grant AI0063107 to S.C. S.C. is funded by a Pew Scholar Award.

Immunological Reviews 2006
Vol. 211: 320–337
Printed in Singapore. All rights reserved

© 2006 *The Authors*
Journal compilation © 2006 *Blackwell Munksgaard*
Immunological Reviews
0105-2896

Summary: The smallpox vaccine consists of live vaccinia virus and is generally considered the gold standard of vaccines, since it is the only one that has led to the complete eradication of an infectious disease from the human population. Renewed fears that smallpox might be deliberately released in an act of bioterrorism have led to resurgence in the study of immunity and immunological memory to vaccinia virus and other poxviruses. Here we review our current understanding of memory T-cell, memory B-cell, and antibody responses to vaccinia and related poxviruses, both in animal models and human subjects. Of particular interest are recent advances in understanding protective immunity to poxviruses, quantifying immunological memory to the smallpox vaccine in humans, and identifying major vaccinia-specific T-cell and B-cell epitopes. In addition, potential mechanisms for maintenance of immunological memory are discussed.

Introduction

Immunological memory following vaccination or infection provides a selective advantage upon re-exposure to the pathogen of interest; individuals undergoing a primary infection will be subject to the full extent of potential morbidity and mortality, whereas those with effective pre-existing immunity (i.e. immunological memory) will be afforded levels of protection that may either prevent initial infection or decrease the severity of disease manifestations. Immunological memory can be maintained by several antigen-dependent means including re-activation of latent/chronic infections or through re-exposure to the particular infectious agent. One of the greatest questions faced by immunologists today is the issue of how immunological memory is maintained in the absence of repeated antigenic boosting events. Vaccinia virus, the agent used in traditional smallpox vaccination, has provided an excellent model for not only studying the duration of immunological memory, but also deciphering the mechanisms underlying long-term memory T-cell, memory B-cell, and antiviral antibody responses. This virus does not spread

systemically or persist in normal healthy individuals (1), so self-boosting by means of a latent infection is not an issue. Although vaccinia viruses may cause isolated zoonotic outbreaks (2–4), it is not endemic in the United States, and with the end of civilian smallpox vaccination in 1972, immunity can be monitored in the absence of re-exposure to vaccinia or other potentially cross-reactive orthopoxviruses (5). Modern smallpox vaccination was first developed by Edward Jenner over 200 years ago (6) and has been extensively studied in terms of virology, pathology, epidemiology, and immunology (7). The vaccinia genome of approximately 200 genes has been mapped, the virus lifecycle has been extensively studied, and with fears that smallpox might be deliberately released in an act of bioterrorism, there has been a renewed interest in the study of immunity and immunological memory to vaccinia and other poxviruses. Analysis of the mechanisms involved in protective immunity against poxviruses have been key to identify antigenic targets of vaccinia virus since development and assessment of novel smallpox vaccines with improved safety profiles depend on a detailed understanding of the mechanisms of immunity. The experimental approaches used in recent studies on poxvirus immunity are also relevant to other human vaccine development efforts.

Kinetics and duration of vaccinia immune responses

Animal models

Inoculation of immunocompetent mice with a vaccine strain of vaccinia virus results in an acute infection that elicits a strong adaptive immune response (8, 9). Many studies have used the neurovirulent Western Reserve strain of vaccinia virus (VV-WR) to elicit more robust immune responses after intraperitoneal or intradermal inoculation (8, 10, 11). In contrast, intranasal inoculation with high doses of VV-WR can result in a lethal respiratory infection (9, 11–14).

Mice make strong antibody responses after vaccinia virus infection. Although low levels of antibody are detected at day 7 postinfection, strong immunoglobulin M (IgM) and IgG responses (including multiple IgG isotypes) are clearly present by day 14 postinfection (15). Antibody titers are higher at 1 month postinfection (10) and are maintained for more than 3 months (16). Antibody responses to vaccinia are almost completely $CD4^+$ T-cell-dependent, with a minor T-cell-independent IgM response (10). Neutralizing antibodies develop with kinetics similar to the overall IgG response (9) and are thought to be maintained long-term. Vaccinia-specific memory B cells are generated after infection (S. Crotty, unpublished data).

Anti-poxvirus antibody responses are also developed after immunization of mice with modified vaccinia virus Ankara (MVA), a highly attenuated candidate smallpox vaccine, but higher doses of immunizing MVA are required (9). Unexpectedly, low doses of MVA appear to elicit T-cell responses in mice in the absence of detectable antibody responses at 1 month postprimary vaccination (14).

The kinetics and duration of vaccinia virus-specific T-cell responses in mice were determined in an extensive study by Ahmed and colleagues (17). Strong $CD8^+$ T-cell responses were detected by day 7 postinfection, which then declined over time and stabilized by day 30, at which point levels of memory $CD8^+$ T cells were maintained stably for more than 300 days postinfection. $CD8^+$ T cells could be detected as early as day 6 postinfection and exhibited both interferon-γ secretion and *ex vivo* cytotoxic T-lymphocyte (CTL) activity. Notably, at the peak of the effector T-cell response, as many as 30% of the $CD8^+$ T cells were specific for vaccinia virus. The magnitude of effector $CD8^+$ T-cell responses in an independent study was comparable (10).

Vaccinia virus-specific $CD4^+$ T-cell responses in mice also peak at approximately 1 week postinfection. The effector $CD4^+$ T-cell response comprises approximately 4–5% of total splenic $CD4^+$ T cells (10, 17), which is comparable to lymphocytic choriomeningitis virus (LCMV) infection (17) and higher than influenza infection (18). Stable numbers of vaccinia virus-specific memory $CD4^+$ T cells were detected from 1 to 7 months postinfection (17). Most effector $CD4^+$ T cells produced interferon-γ, but there was heterogeneity in the population, with some cells producing tumor necrosis factor and/or interleukin-2 (IL-2) (17). The frequency of double cytokine producers and triple cytokine producers increased in the memory $CD4^+$ T-cell compartment (17). This finding is similar to patterns observed for acute LCMV and influenza infections (19–22).

Non-human primates have been used extensively for testing poxviruses as recombinant vaccine vectors delivering heterologous antigens (23–25). Such studies frequently utilized vaccinia virus vectors, but some utilized MVA, canarypox-, or fowlpox-based vectors (23–25). Rhesus macaques (RMs) immunized with vaccinia virus rapidly develop a neutralizing IgG antibody response (26) that is maintained stably for more than 6 months postimmunization (27). RMs also develop a strong $CD8^+$ T-cell response that peaks at approximately 2 weeks postimmunization in peripheral blood (26). The magnitude of the effector $CD8^+$ T-cell response peaked between 0.1 and 1% of $CD8^+$ T cells (26). $CD4^+$ T-cell responses are also elicited, but data on the magnitude and kinetics are limited (26, R.R. Amara, personal communication).

Humans

Strong antipoxvirus antibody responses are elicited in individuals receiving the smallpox vaccine. Neutralizing antibodies against both intracellular mature virion (IMV) and extracellular enveloped virus (EEV) vaccinia virion forms are produced (28–32). Antibody responses are generally not detected by either enzyme-linked immunosorbent assay (ELISA) or neutralization assays before day 10 postimmunization (33–35). Antibody responses can be readily detected on day 14 postimmunization (33–36). Peak antibody levels are present by 1 month postimmunization (R. Ahmed, personal communication). There has long been data suggesting that human antibody responses after vaccination are long lived (5, 7, 37). An extensive cross sectional study showed that peak antibody responses appeared to decline during the first year postvaccination, but antibody levels then stabilized and remained nearly constant for up to 75 years postimmunization (38). Several additional studies have published data corroborating these results (34, 39–42). Neutralizing antibodies are also maintained long-term, with IMV-specific and EEV-specific neutralizing antibody titers being relatively consistent with ELISA titers (29, 38, 39, 42). Anamnestic antibody responses after booster vaccination can be detected as early as day 4 after immunization (33), and rapid recall responses may be important for protection against smallpox.

Vaccinia-specific memory B cells are generated after immunization, and approximately 1% of circulating IgG^+ memory B cells are specific for vaccinia virus at 1–6 months postvaccination (39). Cross sectional analysis of human vaccinees ranging from 4 weeks to 60 years postimmunization indicated that the number of vaccine-specific memory B cells declined by about 90% during the first few months/years postvaccination and then appeared to stabilize, with vaccinia-specific IgG^+ memory B cells maintained for more than 50 years (39).

Humans make strong $CD8^+$ T-cell and $CD4^+$ T-cell immune responses after receiving the smallpox vaccine. Indeed the existence of a delayed hypersensitivity reaction after immunization, was first observed by Jenner in his initial smallpox vaccination studies over 200 years ago (7). Vaccinia-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses in humans were first described 15 years ago (43, 44). More recently, several studies have used quantitative techniques to examine the magnitude and duration of vaccinia-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses. A detailed kinetic analysis of the effector $CD8^+$ T-cell response after vaccination has not been completed, but studies indicate that the effector $CD8^+$ T-cell responses (interferon-γ-producing cells capable of CTL activity) peak at approximately 2 weeks postvaccination (45, 46). Several groups have reported strong $CD8^+$ T-cell responses at 3–5 weeks postvaccination: 0.04–3% of $CD8^+$ T cells are virus specific, as measured by intracellular cytokine standing (38, 45, 47). A slow decline in circulating vaccinia-specific $CD8^+$ T cells was observed thereafter (38, 45, 46). The vaccinia virus-specific $CD4^+$ T-cell response appears to be lower than the $CD8^+$ T-cell response at 2 weeks postvaccination (45) but similar in magnitude to the $CD8^+$ T-cell response at 1 month postvaccination (38, 45, 47).

By performing cross sectional analysis of a cohort of 306 smallpox vaccine recipients, Hammarlund *et al.* (38) demonstrated that the vaccinia virus-specific memory $CD4^+$ T cells are long lived. However, the memory $CD4^+$ T-cell population declined slowly, with a half-life of 8–12 years. These results were corroborated in two additional studies (39, 45). The memory $CD8^+$ T-cell response to vaccinia was also long lived but with an unexpected twist: vaccine-specific memory $CD8^+$ T cells dropped below detection in approximately half of the vaccinees sometime before 20 years postvaccination. The reasons for this observation are unclear, but the preferential survival of $CD4^+$ memory over $CD8^+$ memory have been confirmed in an independent study (45).

The longevity of B- and T-cell memory and antibody responses to vaccinia observed in these human studies is consistent with epidemiological work suggesting that the immunity against smallpox is maintained for decades after vaccination (5, 7, 48, 49). These data on smallpox vaccine-induced B-cell and T-cell memory also have important implications for the longevity of immunity elicited by other vaccines and may be of particular interest for vaccine strategies employing poxvirus-based vectors related to vaccinia virus, such as MVA and canarypox. However, since MVA and canarypox lack the capacity to replicate in most mammalian cells, their ability to generate long-term immunity may not be equivalent to the longevity of immune responses induced by a replicating virus such as vaccinia.

Fine specificities of smallpox vaccine B-cell and antibody responses

Animal models

Antibody responses against vaccinia virus in mice have been identified for at least 10 different proteins by Western blot probing of purified virions or lysate from infected cells (50, 51). Typically, Western blot techniques only identify linear epitopes and not conformational epitopes. Moreover, many vaccinia virus proteins are present in low quantities, which makes it somewhat complicated to perform side-by-

side quantitative analysis of immunodominance. For these reasons, Felgner and colleagues (30) adopted the approach of developing a proteome array of vaccinia virus. Recombinant proteins were expressed from 185 different vaccinia virus genes and used to make an extensive proteome microarray (30). When probed with serum from vaccinia virus immunized mice, the proteome array revealed antibodies directed against 21 different vaccinia virus proteins, 11% of the total vaccinia proteome (30). This strategy was then used to identify vaccinia virus surface proteins of interest for protective immunity (11).

Neutralizing antibodies mainly confer protection through the recognition of structures on the surface of virus particles, and therefore antiviral antibodies directed against the surface of virions are the primary antibodies of interest. Vaccinia virus has more than 20 surface proteins. Importantly, vaccinia virus has two forms of infectious virions, each expressing a different set of surface proteins. As such, an understanding of the virion structures is required to develop knowledge regarding the targets of protective antibodies. The most abundant particle is the IMV, which accumulates in infected cells and is released as cells die (52). IMVs are environmentally stable infectious virus particles and likely represent the principle type involved in transmission between hosts. An alternate morphogenesis pathway is taken by a proportion of IMVs inside infected cells. These immature virions become wrapped in a double membrane from the *trans*-Golgi and are then translocated to the cell surface where the outermost membrane fuses with the plasma membrane (53). These virions may be released from the cell surface as EEVs (54). EEVs are more fragile and less abundant than the IMVs and are considered to be primarily involved in dissemination within the same host rather than transmission between hosts (54–56). Studies have shown that antibodies against either IMV or EEV particles can be protective (11, 56–58). In the context of a vaccine to prevent host-to-host transmission, antibodies against IMV surface antigens might be expected to play a major role in protection. In general, anti-IMV antibodies may protect primarily by neutralizing the virus inoculum, and anti-EEV antibodies may protect primarily by limiting viral spread after infection, though there remains debate as to the relative *in vivo* contributions of antibodies against IMV versus EEV forms of the virion (28).

There are 17 known or predicted IMV surface proteins (*Table 1*) (173). Neutralizing antibodies directed against five of these proteins have been reported in various animal models: L1R, A27L, A17L, H3L, and D8L (50, 58–70). There are six membrane proteins present exclusively on EEVs (*Table 2*), and only four of them have domains exposed on the outside of the membrane: B5R, A33R, A34R, and A56R (54). Neutralizing antibodies have been reported against B5R (56, 57). Antibodies against B5R or A33R are protective in mice (56, 57). EEVs are very fragile, making it difficult to accurately measure neutralizing antibodies against this form of virus. Interestingly, anti-A33R antibody responses were shown to be protective in animal studies (57) before evidence was

Table 1. **Vaccinia intracellular mature virion antibody targets**

VV protein	Function	Neutralizing antibodies shown in animal model	Protection shown in animal model	Neutralizing antibodies shown in humans	References
A9L	Early morphogenesis				156
A13L	Virion maturation				157
A14L	A27 complex				66
A14.5L	Unknown. Affects virulence.				158
A17L	A27 complex	Yes			66
A21L	Entry/fusion apparatus				159
A27L	Cell binding and fusion	Yes	Yes		58, 64
A28L	Entry/fusion apparatus				160
D8L	Cell adhesion	Yes			62
D13L	Early morphogenesis. Primarily found on immature virions.				161–163
E10R	Thiol oxidoreductase				164
F9 (predicted)	Unknown				165
H2R	Entry/fusion apparatus				166
H3L	Cell adhesion	Yes	Yes	Yes	11, 68
I5L	Unknown				167
L1R	Unknown (possible fusion protein)	Yes	Yes		56, 59–61
L5R	Fusion				168

Table 2. **Vaccinia extracellular enveloped virus antibody targets**

VV protein	Function	Neutralizing antibodies shown in animal models	Protection shown in animal models	Neutralizing antibodies shown in humans	References
A33R	Unknown	Yes ('comet tail' inhibition)	Yes		56, 57, 71
A34R	Unknown. Lectin similarities				54
A36R	Actin tail formation. Not surface exposed.				169, 170
A56R	Unknown				54
B5R	Required for EEV membrane wrapping.	Yes	Yes	Yes	31, 56, 57, 171, 172
F13L	Has complement control domain homology. Lipase? Not surface exposed.				54

obtained that anti-A33R antibodies can neutralize EEVs as measured by the 'comet tail' inhibition assay (56, 71). Spread of vaccinia virus strain IHD-J EEV *in vitro* results in characteristic comet tail plaques, and those comet tails can be blocked by the presence of EEV neutralizing antibodies in the culture medium. The difficulty in directly measuring anti-A33R-neutralizing function (in a traditional virus neutralization assay) is because binding of anti-A33R can result in sections of EEV membrane being ripped away *in vitro*, revealing the underlying IMV that is still infectious (71). Alternatively, anti-A33R antibodies may not need to neutralize EEVs directly but may instead block viral replication and/or comet tail formation by other means, such as by targeting virus-infected cells or the release of EEV from infected cells (72). Oddly, in the presence of added complement, anti-A33R antibodies do not inhibit comet tail formation but instead actually enhance comet formation and thereby appear to increase virus spread *in vitro* (71). This experience underscores the complexity of assessing neutralizing antibody functionality in protection from poxvirus infections.

Humans

Humans, like mice, make antibody responses to numerous vaccinia virus proteins after immunization. Five to 13 viral proteins can be detected by Western blot analysis with serum from immunized human subjects (32, 50). Fourteen different vaccinia proteins were detected by probing a vaccinia proteome microarray with serum from immunized humans (30). IMV surface proteins, EEV surface proteins, virion core proteins, and soluble proteins are all recognized, demonstrating that the human anti-vaccinia antibody response is broad (30, 32).

Previous studies have identified a correlation between preexisting serum neutralizing antibody titers and protection against contracting smallpox (73, 74). An important goal in poxvirus research is to identify key antigenic targets of vaccinia virus that are recognized by antibodies in immunized humans and are critical for protection against disease. As part of that effort, H3L was identified as the first known target of human IMV neutralizing antibodies (11). H3L protein is a viral receptor involved in cell adhesion, and the H3L gene is strongly conserved among the orthopoxviruses. For example, the H3L proteins from vaccinia virus and smallpox share 97–98% amino acid identity, which may contribute to the observed cross-protection against variola afforded by vaccinia immunization (75). Antibodies to the orthologs of H3L are also seen after infection with other orthopoxviruses, including orf virus, capripox, and fowlpox (76–78). Regarding EEV, only B5R has been reported as a target of human EEV neutralizing antibodies (31), and anti-B5R antibodies appear to be the dominant contributor to EEV neutralization in humans (31) (*Table* 2). B5R protein is also highly conserved between vaccinia and smallpox, with approximately 93% sequence identity (75).

The antigenic epitopes of vaccinia-specific human memory B cells have not been identified to date. Identification of the specificities represented within the memory B-cell pool may provide predictive power regarding protection from a smallpox exposure, as the rapid anamnestic antibody response post-infection is primarily driven by the size of the memory B-cell compartment and the composition of vaccinia virus neutralizing antibody specificities represented therein. Individuals possessing a high frequency of memory B cells specific for major neutralizing antibody targets of vaccinia virus would be expected to be better protected from smallpox than individuals with a memory B-cell pool dominated by specificities for nonprotective vaccinia antigens. Differences in immunodominance profiles within the memory B-cell compartment may be important characteristics of candidate smallpox vaccines.

Fine specificities of smallpox vaccine T-cell responses

Animal models

Use of vaccinia-infected target cells has made it feasible to measure vaccinia virus-specific $CD8^+$ T-cell and $CD4^+$ T-cell

responses without knowledge of major histocompatibility complex (MHC) class I and MHC class II vaccinia epitopes (17). This ability has been particularly important, since the size of the vaccinia virus genome has long precluded efforts to identify virus-specific MHC I and MHC II epitopes, either in animal models or humans. Nevertheless, there are clear limits to the immunological analysis that can be done in the absence of knowledge of MHC I and MHC II epitopes. For example, studies with vaccinia (79), influenza (80, 81), and LCMV (82, 83) have shown that epitope-specific T cells can demonstrate strikingly different functional and/or phenotypic profiles. Moreover, peptide-specific analysis of T-cell responses elicited by different smallpox vaccines (vaccinia virus versus MVA versus LC16m8) will be necessary to determine if these vaccine candidates induce comparable T-cell epitope specificities that are conserved in smallpox.

Yewdell and colleagues (84) recently identified five MHC I $CD8^+$ T-cell epitopes in C57BL6 mice by generating an expression library containing each of the >200 vaccinia virus predicted open reading frames. The immunodominant $B8R_{20-27}$ epitope identified by this strategy was recognized by approximately 10% of $CD8^+$ T cells at the peak of the response to vaccinia virus. In contrast, each of the other four MHC I epitopes were recognized by <4% of $CD8^+$ T cells. The five epitopes identified in this study did not exhibit any obvious commonality, except that each of these viral peptides binds MHC I with nanomolar or subnanomolar affinity, suggesting that the high binding affinity may be an important criterion determining the targets of $CD8^+$ T-cell responses to vaccinia virus. Of interest, the immunodominance profile and magnitude of the $CD8^+$ T-cell response varied depending on the poxvirus strain and species used and the route of infection or immunization used (84). The expression library strategy was impressively successful in identifying epitopes, but it is presumed that not all C57BL6 MHC I epitopes were found. Indeed, using a large-scale bioinformatics and peptide synthesis screening strategy, Sette and colleagues (A. Sette, personal communication) have identified additional vaccinia virus MHC I epitopes in C57BL6 mice. No vaccinia virus-specific MHC II epitopes have been reported.

Humans

As mentioned above, the large size of vaccinia virus makes it difficult to identify antigenic determinants. This identification becomes particularly difficult when trying to identify human T-cell epitopes. There are over 100 human leukocyte antigen (HLA) alleles (85) and a vast array of potential vaccinia-specific $CD8^+$ T-cell determinants (86). Given such complexity, three strategies have been employed to identify human $CD8^+$ T-cell epitopes of vaccinia virus. First, several laboratories have utilized predictive computational algorithms to identify vaccinia peptides that bind a specific HLA allele (e.g. HLA-A2, the most common allele in the US population), and then screened those predicted peptides in HLA transgenic mice infected with vaccinia virus. This strategy has resulted in the identification of a total of 25 different HLA-restricted vaccinia epitopes by three independent studies (86–88). Of those 25 epitopes, almost all were from genes encoding proteins larger than 100 amino acids (86). In addition, approximately half of the determinants were encoded by early expressed proteins and half by late expressed viral proteins (86). This outcome appears to be different from herpesvirus infections, for which the majority of $CD8^+$ T-cell determinants are from early expressed viral proteins. For one identified vaccinia epitope, it was shown that peptide immunization could elicit a protective $CD8^+$ T-cell response in HLA transgenic mice (87), whereas a second epitope appeared to be non-protective in mice (88). Therefore, it will be important to perform parallel experiments with multiple epitopes to determine whether all epitopes are equally valuable in protection or whether epitopes against certain viral proteins (or classes of viral proteins, such as early expressed proteins) are the most important for protective immunity. More generally, a caveat of HLA transgenic mouse work is that currently only one of the 25 identified vaccinia virus epitopes has been shown to be recognized by $CD8^+$ T cells from vaccinated humans (88).

The second strategy employed has bypassed problems of computational predictions by directly cloning out $CD8^+$ T-cells specific for vaccinia virus from immunized humans. Two HLA-A2 epitopes were identified in this manner. One caveat to this approach, however, is that major/immunodominant epitopes are identified, but it is difficult and labor-intensive to measure an overall T-cell response comprised of multiple specificities or one which contains many subdominant epitopes (46). Synthetic peptide combinatorial libraries (89) may also provide an effective approach to identifying and characterizing more vaccinia-specific T-cell epitopes. This third strategy has been employed in a massive screen of more than 6000 peptides predicted by bioinformatics approaches to have good HLA-binding characteristics. These candidate peptides were tested using peripheral blood samples from 58 immunized human donors (90). Forty-eight epitopes to vaccinia virus were identified in this manner. The epitopes were from 35 different vaccinia virus proteins, representing almost 20% of the predicted open reading frames in the genome (90). For a given individual, summing the

individual peptides specific responses could account for approximately one-third of the response observed against vaccinia-infected target cells. This finding suggests that the human $CD8^+$ T-cell response to vaccinia virus is broad and usually directed to more than six different epitopes. In addition, there was extensive heterogeneity among determinants recognized by individuals with a given HLA allele, suggesting that the interactions between HLA alleles (and other genetic or environmental factors) result in many different patterns of vaccinia virus epitopes recognized by human $CD8^+$ T cells.

Protective immunity against orthopoxviruses

The correlates of protective immunity against orthopoxviruses such as vaccinia, monkeypox, and smallpox have long been debated. Much of the conflicting data in the literature comes from the use of many different poxvirus species, strains, and routes of infection, and a failure to clearly distinguish the importance of different arms of the adaptive immune system in primary immune responses versus secondary (recall) immune responses in primed or vaccinated animals. In mouse models of vaccinia infection, extensive studies have shown that passive immunotherapy with immune serum or monoclonal antibodies are protective (11, 28, 56, 91). In studies wherein both T-cell and antibody responses were compared (10, 14), it was revealed that antiviral antibody could protect mice efficiently even if $CD4^+$ or $CD8^+$ T cells were depleted prior to challenge. However, in B-cell-deficient or MHC II-deficient mice, which are unable to elicit effective antibody responses, strong antiviral T-cell responses played an important role in protecting against disease following viral challenge. For highly pathogenic viruses such as ectromelia (mousepox), the requirements for protective immunity again pointed to the humoral immune response, but with the added observation that antiviral antibody and $CD8^+$ T cells play complementary roles with the most efficient protection provided by a combination of pre-existing humoral and cell-mediated immunity (92).

The murine models of orthopoxvirus infection indicated roles for both cellular and humoral arms of the immune response in protection against disease and/or lethal infection. To clarify this issue in an animal model that most closely resembles human smallpox infection, a recent study examined the role of vaccine-induced antibody and T-cell responses in protection of non-human primates from lethal monkeypox infection (27). In these studies, vaccinia-immune RMs were depleted of $CD4^+$ or $CD8^+$ T cells at the time of monkeypox challenge. Neither T-cell depletion strategy reduced protection against lethal challenge. This finding indicated that the memory T cells were not necessary for control of the monkeypox infection. In contrast, when $CD20^+$ B cells were depleted at the time of vaccination to block induction of neutralizing antiviral antibody responses, three of the four treated animals died following monkeypox challenge. Interestingly, the one animal that survived monkeypox challenge was also the only animal in which the anti-CD20 depletion was suboptimal, and this animal mounted a low but detectable antiviral antibody response. These studies demonstrated that antiviral antibodies were necessary for protective immunity against monkeypox, but to demonstrate that antibody was sufficient for protection, the investigators administered human vaccinia immune globulin (VIG) to unvaccinated RMs 4 days prior to monkeypox challenge. The results were impressive; although the animals developed skin lesions (i.e. pocks) in a dose-dependent manner with an inverse relationship to the amount of VIG administered, they were still fully protected from lethal infection. This study demonstrated that the smallpox vaccine-induced antibody responses were both necessary and sufficient for protection against lethal monkeypox infection, an important benchmark in the immunobiology of orthopoxvirus research.

Based on the severe complications following smallpox vaccination of children with genetic T-cell deficiencies, it was initially believed that cellular immunity played the most important role in protective immunity in humans (7). However, these cases represented primary poxvirus infections; following vaccination, strong antiviral antibody responses are likely to be the main effector mechanism responsible for protection against secondary infection. In clinical studies, there is a preponderance of data demonstrating the important role of antiviral antibodies in protecting against smallpox (5). VIG was commonly used to treat rare but severe complications following smallpox vaccination (93). VIG reduced the spread of smallpox outbreaks when administered at the same time as smallpox vaccination to smallpox contacts (93–95) and alleviated disease severity in anecdotal cases of smallpox infection (96). Moreover, high neutralizing antibody titers were associated with protective immunity against smallpox infection (74, 97), even though there is no correlation between long-term antibody titers and T-cell memory (39, 98), thus ruling out the possibility that high antibody titers were simply a biomarker for high pre-existing T-cell memory. A most impressive study demonstrating the utility of passive immunotherapy was published in 1941 (99). In this study, a smallpox outbreak initially claimed the lives of three out of 10 patients. When patient care was expanded to include administration of high-titer smallpox-specific convalescent

serum at the first signs of disease, the mortality rate dropped to 0%(0 deaths out of 250 subsequent smallpox infections reported). Severe disease was not only modified, but the authors also noted that patients experienced fewer smallpox scars after recovery. These results together with the confirmatory evidence obtained in several animal models illustrate the importance of humoral immunity in protection against orthopoxvirus infections. These observations are important for future vaccine design and are especially relevant in contemporary populations with an ever-increasing number of individuals lacking immunity to orthopoxviruses, due to being born since the cessation of routine smallpox vaccination over 30 years ago.

Role of $CD4^+$ T cells in the establishment of humoral immunity

Our understanding of $CD4^+$ T-cell help in generating and maintaining humoral immunity following acute viral infection continues to evolve. By definition, induction of T-dependent (TD) humoral immune responses requires $CD4^+$ T-cell help (100–102). However, one can segregate TD humoral immune responses into at least three distinct phases: induction, maintenance, and reactivation/anamnestic responses following secondary challenge. Interestingly, $CD4^+$ T cells appear to play different roles depending on the antigen and the phase of the elicited immune response in question.

$CD4^+$ T-cell depletion studies demonstrate that the induction of strong antibody responses requires an intact $CD4^+$ T-cell compartment (103). $CD4^+$ T-cell help is heavily mediated by CD40:CD40L interactions (104–106). The induction phase is rather short-lived, and as the time span increases between immunization and $CD4^+$ T-cell depletion, the mounted humoral response becomes stronger, indicating a diminished requirement for $CD4^+$ T-cell help (101–103) (*Fig. 1*). If $CD4^+$ T-cell depletion is delayed until approximately 3 weeks after immunization, then essentially normal antigen-specific antibody responses are observed (103). Similar results were noted in cases of retrovirus-induced loss of $CD4^+$ T-cell numbers/function. Following vaccinia infection of simian immunodeficiency virus-infected RMs, animals with $CD4^+$ T-cell counts below 300 cells/mm^3, i.e. approaching simian acquired immunodeficiency syndrome (AIDS), were much less capable of mounting protective neutralizing antibody responses than animals with $CD4^+$ T-cell counts of >300 cells/mm^3 (107). Additional evidence for the importance of $CD4^+$ T cells in controlling primary vaccinia infection comes from human immunodeficiency virus (HIV)-infected patients. Although approximately 300 HIV-infected military

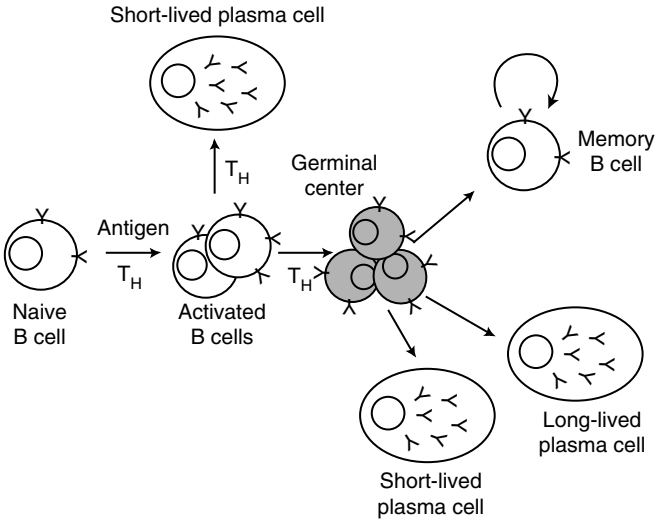

***Fig. 1.* Model of B-cell differentiation during an antiviral immune response.** Following antigenic stimulation, naïve B cells undergo clonal expansion and form clusters of activated B cells known as extrafollicular foci. These activated B cells can either differentiate into short-lived plasma cells, or they can migrate into the follicle and initiate a germinal center reaction. After proliferation and affinity maturation, germinal center B cells produce both short- and long-lived plasma cells that produce high affinity antibodies, and memory B cells that have high affinity B-cell receptors. $CD4^+$ T-cell help is required during the induction stages of the B-cell response but is not required for maintenance of memory. See main text for details. T_H = $CD4^+$ T-cell help.

personnel have likely received smallpox vaccinations with replication-competent vaccinia virus (108), only one case of life-threatening infection was noted, and this individual had apparently progressed to AIDS with $CD4^+$ T-cell counts below 25 cells/mm^3 by the time he was hospitalized at 2.5 weeks postvaccination. This individual was treated with VIG and recovered, but about 7 months later he succumbed to an unrelated AIDS-associated disease (109). In a separate study, out of eight AIDS patients immunized with a recombinant strain of vaccinia virus, three died from viral complications (110). These three patients exhibited the lowest $CD4^+$ T-cell counts (<50 cells/mm^3), suggesting the virus-associated complications arose from a lack of cellular immunity and/or ability to mount an effective TD antibody response. Other rare but severe and life-threatening complications stemming from smallpox vaccination have provided additional insight into this aspect of the immune response. Vaccinia necrosum, a progressive infection caused by uncontrolled spread of vaccinia, leads to tissue destruction and nearly 100% mortality in individuals that are genetically deficient in cellular immunity. In addition to T-cell deficiencies, these patients also exhibit low or negligible virus-specific antibody titers following vaccinia infection, indicating that lack of T cells resulted in abrogation of both cellular and humoral immunity (93).

Fortunately, administration of VIG was able to save approximately 80% of these children, even in cases where the patients were genetically incapable of mounting antiviral T-cell responses. Children lacking humoral immunity (i.e. agammaglobulinemic or hypogammaglobulinemic) are known to be somewhat less susceptible to severe viral infections as children lacking cellular immunity. These 'experiments of nature' have often been cited as evidence that T cells are the most important component of protective immunity against viral infections. However, this evidence is somewhat misleading. In the absence of humoral immunity, the T-cell response is largely intact (and potentially capable of compensating for antibody deficiencies), whereas in cases of T-cell dysfunction, both humoral and cellular arms of the immune response are impaired. Thus, it is not surprising that the T-cell deficiencies result in the highest susceptibility to microbial infections, but these findings should be interpreted within the proper context. T-cell immunity is most important during primary acute viral infections for directly controlling viral replication as well as for helping to establish the humoral immune response. However, protection against reinfection is primarily antibody-mediated for orthopoxvirus infections, and T cells play a secondary role in vaccine-induced protective immunity (27).

Studies have demonstrated that maintenance of B-cell memory and long-term antibody production can occur in the absence of continuous $CD4^+$ T-cell help (103, 111). In one case, $CD4^+$ T cells were continuously depleted for up to 6 weeks without any substantial effect on B-cell memory (103), and in another case, memory B cells were adoptively transferred into T-cell-deficient hosts and survived for at least 90 days in the absence of a detectable $CD4^+$ T-cell population (111). An interesting new perspective on this topic was described when it was found that antiviral antibody responses are defective in *SAP*-deficient mice (112). *SAP*, a gene linked to fatal lymphoproliferative disease often marked by low IgG antibody titers, appears to be important in controlling long-term humoral immunity. *SAP* knockout mice showed similar antibody titers and numbers of antibody-secreting cells to wildtype mice at early stages of a TD antiviral immune response. However, memory B-cell development was delayed, the absolute number of memory B cells was reduced by approximately 100-fold, and the number of germinal centers was reduced by approximately 10-fold. Bone marrow plasma cells were also greatly reduced, and antibody responses declined with rapid kinetics, indicating that long-lived plasma cells were not elicited. This defect was not intrinsic to the B cells, since adoptive transfer experiments demonstrated that SAP-deficient B cells are normal, whereas SAP-deficient $CD4^+$ T cells have a defect in providing appropriate B-cell help despite expressing appreciable levels of CD40L (112). These data indicate that the maintenance of antibody production and the development of long-lived plasma cells may be dictated at or near the intersection between the induction phase and maintenance phase of the humoral immune response.

Clinical studies involving smallpox vaccination have provided further insight into the role of $CD4^+$ T-cell memory requirements for maintaining long-term humoral immunity. As mentioned earlier, there is no statistical correlation between $CD4^+$ T-cell memory and long-term serum antibody levels (38, 39). If serum antibody responses or B-cell memory were directly linked to requirements of $CD4^+$ T-cell help, then one would have expected these arms of the humoral immune response to correlate with $CD4^+$ T-cell memory and to decline with similar kinetics as that observed with the antiviral $CD4^+$ T-cell response. Since this is not the case, it provides compelling albeit indirect evidence that $CD4^+$ T-cell help is unlikely to be required during the maintenance phase of the humoral immune response against vaccinia. A lack of correlation between T-cell memory and humoral immunity also has been noted in other studies. One report investigated the maintenance of cellular and humoral immunity following natural infection or vaccination against measles virus (MV) in 56 individuals, ranging from 23 to 47 years postexposure or 1–34 years postvaccination (113). In these studies, a significant decrease in $CD4^+$ T-cell memory at time points >21 years postvaccination was observed, whereas a similar decrease was not seen in anti-MV antibody titers. More importantly, when comparing the frequencies of MV-specific $CD4^+$ (or $CD8^+$) T cells with MV-specific antibody titers, no correlation was observed (113). This finding reiterates the results seen with the vaccinia model, suggesting that T-cell memory and antibody responses are not linked and are subjected to different survival pressures *in vivo*. Another model for investigating the maintenance of antibody levels after the loss of T-cell help can be found in HIV patients who lose $CD4^+$ T cells as they progress towards AIDS. Several studies have investigated the maintenance of serum antibody levels throughout the progression of AIDS, and the findings indicate that the pre-existing antibody response to many antigens remains largely unaltered (114, 115). This point again emphasizes that while T-cell help is critical during initiation of humoral immunity, the persistence of the ensuing response does not appear to require continual antigen-specific T-cell – B-cell interactions.

The role of $CD4^+$ T-cell help following reactivation of memory B cells during anamnestic antigen-specific immune responses appears to be more complex. In an early study measuring hapten-specific antibody responses [(4-hydroxy-

3-nitrophenyl)acetyl (NP)-conjugated chicken gammaglobulin], the investigators noted that $CD4^+$ T-cell help was not required for maintenance of B-cell memory but was required for effective anamnestic responses following secondary challenge with soluble antigen (103). Interestingly, the near absence of a secondary antibody response to soluble antigen in $CD4^+$ T-cell-depleted mice could be partially recovered if a higher dose of antigen was administered with alum as an adjuvant. Similar results were obtained in another model system in which B-cell epitopes were covalently attached to different carrier proteins for primary and booster vaccination (116). Booster vaccination with adjuvant resulted in robust anamnestic responses in the absence of help via memory $CD4^+$ T cells. In more recent studies, it was noted that memory B cells could not respond to soluble viral antigen [purified human cytomegalovirus (hCMV) glycoprotein B (gB)] in the absence of $CD4^+$ T-cell help (111). However, if mice were challenged with intact but non-replicating hCMV virions containing gB, then $CD4^+$ T-cell help was no longer required for strong gB-specific anamnestic responses to be mounted. Histology showed that the adoptively transferred memory B cells remained outside of germinal centers and localized near the marginal zone after antigenic challenge. Together, these studies show that the requirement for $CD4^+$ T-cell help during anamnestic humoral immune responses is not absolute. Secondary antibody responses to soluble antigens require $CD4^+$ T-cell help, whereas antigens mixed in adjuvant or presented in particulate form (such as the surface of an intact virus) no longer require $CD4^+$ T cells for rapid and vigorous antibody responses. This phenomenon may help prevent autoimmune responses to soluble self-antigens, since host $CD4^+$ T cells are tolerant to self due to negative selection in the thymus. Alternatively, circumventing an absolute requirement for T-cell help under certain circumstances may allow rapid recall responses to be mounted against foreign antigens in the proper context (i.e. intact virus, bacteria, or other microbe/parasite) without necessarily waiting for cognate cell-to-cell interactions with antigen-specific $CD4^+$ T cells.

The T-cell requirements for anamnestic memory B-cell responses in human subjects remain to be formally determined. Perhaps future experiments with the vaccinia model of smallpox vaccination could provide insight into this question. For instance, it is possible that subjects with the highest pre-existing $CD4^+$ T-cell memory will mount the strongest anamnestic responses following revaccination. Alternatively, if $CD4^+$ T-cell memory is not required for rapid memory B-cell activation against a replicating orthopoxvirus, then there will be a greater correlation between pre-existing B-cell memory and magnitude of secondary antibody responses than between pre-existing $CD4^+$ T-cell memory and secondary antibody responses.

Mechanisms of maintaining memory T cells

During the past decade, major advances have been made in identifying antigen-independent mechanisms of maintaining immunological memory (117–120). From a number of studies, it is now clear that memory $CD4^+$ T cells and memory $CD8^+$ T cells can persist in the absence of antigen (reviewed in 5, 37, 117, 120). It has also been shown that murine memory $CD8^+$ T cells are not static. They regularly undergo homeostatic proliferation to replenish their numbers, and this proliferative renewal does not require stimulation with antigen or MHC I (121–126). In mice, memory $CD8^+$ T cells are stably maintained, while $CD4^+$ memory T-cell numbers appear to slowly decline (127). In contrast, following multiple heterologous infections, $CD8^+$ T-cell memory appears to decline, whereas $CD4^+$ T-cell memory is more stably maintained under these conditions (128). Much recent work has been focused on the role of cytokines in maintaining T-cell memory. IL-15 and IL-7 are important for the maintenance of memory $CD8^+$ T cells and memory $CD4^+$ T cells (129–133). What triggers the slow homeostatic proliferation that maintains murine memory T cells? One hypothesis is that memory $CD8^+$ T cells undergo homeostatic proliferation when they migrate through the bone marrow, where there are high concentrations of IL-15 and other proliferation and survival signals (134, 135).

Studies of vaccinia virus-specific immunity following smallpox vaccination show that memory T-cell responses are long-lived in the absence of re-exposure to infectious virus (38, 39, 45) but declined with a half-life of 8–15 years (38, 39). Since the memory T cells specific for vaccinia virus are long-lived in the absence of antigenic re-exposure, one hypothesis is that these cells are maintained by a homeostatic proliferation mechanism similar to that observed in mice, but this possibility remains to be formally demonstrated in humans.

Mechanisms of maintaining memory B cells

In humans, vaccinia-specific memory B cells can be detected for 60 years after vaccination (39). This finding indicates that human memory B cells can be maintained for life in the absence of antigenic re-exposure. Smallpox vaccine-specific memory B-cell levels appear to be stable from 10 to 60 years postvaccination (39), indicating that the antigen-specific memory B-cell population is maintained by robust

mechanisms. Memory T cells in humans slowly decline with a half-life >10 years. So why would memory B cells not undergo the same decline? One possibility is that memory B cells do decline over time, but this rate may be too slow to be measured by current methods. More studies will be needed to address this possibility. Alternatively, it is conceivable that memory B cells possess more robust DNA repair systems, such that over time they are more competent to repair damage from environmental insults and thereby maintain their proliferative potential. Although speculative, this idea could be tied to a developmental difference between mature B and T lymphocytes: mature B cells maintain the ability to upregulate extensive DNA repair programs, since they possess the ability to undergo recombination and somatic hypermutation (136).

There are four proposed mechanisms for the maintenance of human memory B cells; the first is antigenic depot. This is the classic model of antigen-dependent immunological memory. It is well described that follicular dendritic cells bind antigen and present antigen to B cells in germinal centers. This antigen presentation is generally agreed to be a key component of the affinity maturation and selection process within the germinal center, although this point is debated (137, 138). It has been proposed that after the generation of memory B cells, the memory cells are maintained by periodic re-encounter with antigen presented long-term by follicular dendritic cells (139). However, essentially all proteins have a finite lifespan with a half-life in the range of hours to weeks. Proteins and protein structure are damaged by environmental conditions, proteases, and interactions with other proteins and presumably are consumed during memory B-cell activation, antigen uptake, and presentation to $CD4^+$ T cells. Therefore, retention of biologically relevant levels of antigen by follicular dendritic cells may only be expected to occur for a period of weeks to months before exhaustion of the antigen depot. Therefore, persisting antigen is highly unlikely to maintain a memory B-cell population for more than 50 years. An exception is the case of replicating antigen, such as a latent herpesvirus infection. As discussed earlier, vaccinia virus causes an acute infection in humans and does not persist (1). In addition, the observation that $CD4^+$ and $CD8^+$ T-cell memory declines over time is further evidence against the possibility of viral persistence, since it is not maintaining the memory T-cell populations in concert with memory B cells and antibodies.

In mice, the longevity of memory B cells in the absence of antigen has long been controversial (reviewed in 37, 140). A transgenic approach utilized by Rajewsky and colleagues has largely settled the matter (141). Rajewsky and colleagues (141) engineered mice with a genetic switch, such that memory B cells expressing an NP-specific B-cell receptor (BCR) could be changed *in vivo* to instead express a phycoerythrin (PE)-specific BCR. By doing so, they could track the PE-specific B cells (which have never seen PE antigen), and they observed that the cells persisted in an antigen-independent manner just as well as other memory B cells (141).

The second proposed mechanism for the maintenance of human memory B cells is stimulation by cross-reactive environmental or self-antigens. B cells are stimulated by direct interaction of the BCR with antigen, and the specificity of a BCR is not absolute: cross-reactivity is observed. Therefore, it is possible that vaccinia virus-specific memory B cells (or any memory B cells) are maintained long-term by intermittent interaction with environmental antigens (i.e. allergens, food products, unrelated pathogens). This hypothesis is exceptionally difficult to test. On a related topic, Rajewsky and colleagues (142) have shown that mature naïve B cells must constantly maintain BCR expression for survival, presumably due to a need for tonic signaling through the BCR. Tonic signaling may or may not be dependent on low affinity binding of the BCR to self-antigens. If the signaling is dependent on self-reactivity, memory B-cell BCRs may also have a requirement for low-grade self-cross reactivity. Alternatively, memory B cells may have different requirements for BCR stimulation and may no longer require antigen for their survival or homeostatic proliferation. This mechanism would be similar to the differential requirements observed between naïve T cells and memory T cells for stimulation through the TCR (123).

The third proposed mechanism for the maintenance of human memory B cells is bystander polyclonal activation. This model is based on the observation that memory B cells stimulated *in vitro* with Toll-like receptor (TLR) ligands or select polyclonal activators will undergo several rounds of proliferation and differentiation into plasma cells, whereas naïve B cells will not (143). Based on this hypothesis, Lanzavecchia and colleagues (143) proposed that memory B cells will proliferate *in vivo* in response to irrelevant infections. This model is mechanistically plausible but seems unlikely, because immunological memory would have to rely on bystander activation via near continuous heterologous infections. In mice housed under specific pathogen-free conditions, serum antibody responses are maintained for the life of the animals (144, 145). Adoptive transfer experiments involving cotransfer of hCMV- and tick-borne encephalitis virus (TBEV)-specific memory B cells into T-cell-deficient hosts showed that only when the correct antigen was

introduced would an appropriate antigen-specific B-cell response occur (111). Together, these findings indicate that polyclonal non-specific activation of memory B cells *in vivo* is unlikely to be involved in maintaining humoral immunity in mice. This hypothesis requires further independent analysis in human subjects, and it will be important to directly measure the *in vivo* proliferation and maintenance of populations of antigen-specific memory B cells before, during, and after a number of heterologous infections and/or vaccinations.

Programmed homeostatic proliferation is the fourth proposed mechanism for the maintenance of human memory B cells. This hypothesis draws from the known mechanisms of $CD8^+$ T-cell memory maintenance in mice (117, 129). As mentioned above, murine memory $CD8^+$ T cells accomplish prolonged maintenance by proliferation primarily via IL-15 signals. Therefore, antigen-specific memory B cells may maintain themselves by a programmed homeostatic maintenance involving intermittent proliferation triggered by certain autocrine/paracrine cytokines or other as yet unknown factors. However, it appears clear that $CD4^+$ T-cell help is not an absolute requirement for maintaining memory B-cell numbers, as discussed earlier.

Mechanisms of maintaining antibody levels

What about plasma cells, the effector cells of B-cell immunity? Serum antibody levels are maintained by constant antibody production from plasma cells. There are different populations of plasma cells with a range of potential half-lives (146, 147). Long-lived plasma cells are a central component of immunological memory, as these cells are largely responsible for the long-term continuous secretion of antibody. In contrast to memory B cells, plasma cells are terminally differentiated and cannot be stimulated by antigen to either divide or increase their rate of antibody production. At early time points following vaccination, short-lived plasmablasts/plasma cells appear to have a half-life of a few days (148–150). Long-lived plasma cells survive for extended periods, with a half-life of 3–4 months in mice (144, 146, 151) with some plasma cells surviving up to 550 days (i.e. 1.5 years) following depletion of the memory B-cell compartment by 800 RAD of γ irradiation (144).

Many human vaccines induce serum antibody responses that persist for decades (152), and therefore vaccine-specific plasma cells must be present for decades. However, most reports of durable antibody responses in humans are plagued by questions about the potential of intermittent re-exposure to the antigen (e.g. live measles/mumps/poliovirus vaccines, etc.). As described above, the smallpox vaccine is an excellent opportunity to resolve this issue, since routine smallpox vaccination in the USA was discontinued over 30 years ago. Hammarlund *et al.* (38) demonstrated that antibody responses after smallpox immunization were maintained for up to 75 years, with similar data published in other independent studies (39–41). So then, how is antibody production maintained for 60+ years? The bulk of ongoing antibody production is likely maintained by long-lived plasma cells. There are two main theories for how numbers of antigen-specific long-lived plasma cells are sustained for years after vaccination (*Fig.* 2): (i) replenishment of long-lived plasma cells from memory B cells and (ii) intrinsic plasma cell longevity.

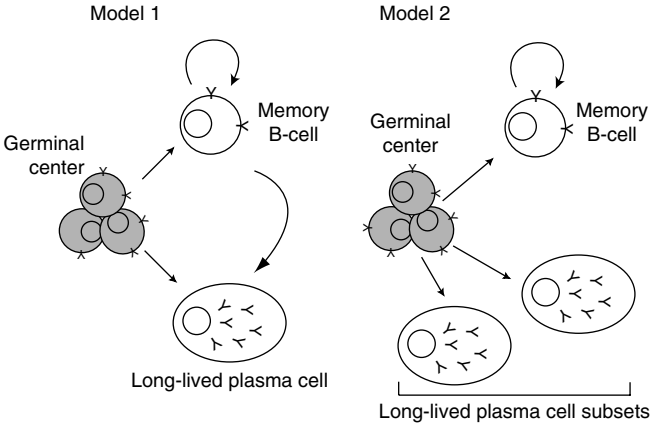

***Fig.* 2. Two models of long-term antibody maintenance in the absence of re-exposure to virus.** Model 1: Long-lived plasma cells may be replenished by proliferation and differentiation of memory B cells. Model 2: There may exist distinct populations of long-lived plasma cells that survive for different amounts of time, including a subpopulation of plasma cells that may survive for several years or even decades. See main text for details.

The first theory for how numbers of antigen-specific long-lived plasma cells are maintained is replenishment of long-lived plasma cells from memory B cells. Plasma cells are terminally differentiated and under normal circumstances are unable to proliferate. Therefore, repopulation of their numbers is thought to require proliferation and differentiation of memory B cells into antibody-secreting plasma cells. As such, this hypothesis posits that intermittent differentiation of memory B cells to long-lived plasma cells occurs and replenishes the plasma cell compartment (*Fig.* 2). In each of several variations of this model, the replenishment of the plasma cells is proposed to occur at the same time as replenishment of the memory B cells. Therefore, the mechanisms proposed for triggering intermittent memory B-cell to plasma cell differentiation are the same as described above for memory B-cell maintenance.

If this model is accurate, there should be a correlation between memory B-cell numbers and plasma cell numbers (or serum antibody levels). Crotty *et al.* (39) showed that there was a positive correlation between anti-vaccinia memory B-cell frequency and anti-vaccinia serum antibody levels. Bernasconi *et al.* (143) showed a similar positive correlation for other antigens using a related technique. However, two other studies found no correlation between memory B-cell numbers and serum antibody titers (153, 154), suggesting that more studies are necessary to determine the relationship between memory B cells and plasma cell populations.

The second theory involves intrinsic plasma cell longevity. This hypothesis is under discussion and is based on the assumption that a subset of plasma cells may survive for decades in the absence of replenishment by the memory B-cell compartment (5, 146). It is known that there are several types of terminally differentiated cell types in the body that survive for decades without replenishment (e.g. many neuronal cell types). Therefore, this model hypothesizes that once a long-lived plasma cell is produced and homes to specific sites such as the bone marrow, the plasma cell may survive for decades without requiring replenishment from the memory B-cell pool. In this model, memory B-cell numbers could correlate with plasma cell numbers. However, this correlation would not be a requirement for sustaining stable serum antibody levels, because they would be discrete and independently regulated cell populations. It is important to point out that not all plasma cells are long lived. During the early stages of an immune response when proliferation and somatic hypermutation are occurring, plasma cells are most likely to be short lived (5, discussion in 144). However, at later stages of the humoral immune response, a subpopulation of proliferating plasma cell precursors will be selected into the long-lived pool of plasma cells with an extended lifespan (*Fig.* 2). If all plasma cells had the same lifespan and were long lived starting at the initiation of the humoral immune response, then there would be a preponderance of low-affinity antigen-specific plasma cells and the potential for autoreactive plasma cells with a dangerously long lifespan to be generated. However, with selection for long-lived plasma cells occurring at later stages of the immune response, only the most effective, high-affinity plasma cells will be maintained long-term. In mice, a subpopulation of plasma cells may survive for longer than 1 year in the absence of repopulation by memory B cells (144). In humans, plasma cells may have a similar lifespan, or they may be longer lived, since humans have a lifespan that is far greater than that observed in mice. Preliminary information has been gained in at least one clinical study (155). In these experiments, peripheral B cells (presumably including memory B cells) were effectively depleted by administration of the anti-CD20 antibody rituximab for treatment of rheumatoid arthritis. Remarkably, serum antibody levels against tetanus toxoid and pneumococcal capsular polysaccharide remained stable for greater than one year after B-cell depletion. However, this study is not without caveats; only two representative patients were followed extensively, the vaccination history of the patients was unclear, and the half-lives of antigen-specific serum antibodies were not determined. Antigen-specific memory B-cell numbers were not directly quantitated either before *in vivo* depletion or after the subject's peripheral B cells were reconstituted months later, so it is unclear what proportion of the memory B-cell pool was specifically removed by this therapy or if memory B-cell numbers rebounded after cessation of therapy. Nevertheless, based on the high expression levels of CD20 on memory B cells, this study provides preliminary evidence indicating that CD20-negative antibody-secreting cells (i.e. plasma cells) are able to maintain stable serum antibody titers for a substantial period of time in humans. More clinical studies are needed to determine the half-life of plasma cells in humans and to determine whether plasma cells of different specificities may be imprinted with different lifespans depending on the nature of the antigenic exposure involved during the initiation or development of the long-term humoral immune response. Further quantitation of memory B cells and serum antibody titers to a number of different replicating and non-replicating antigens will also be required in order to better understand if plasma cell numbers are directly maintained by memory B cells or whether these are instead two independent arms of the humoral immune response. Resolution of these different models of antibody maintenance awaits further study.

Summary

The smallpox vaccine is often considered the gold standard of vaccines, since it is the only one that has led to the total eradication of a disease from the human population. Vaccinia virus has been invaluable both as a human vaccine and as a model for understanding antiviral immune responses. Much progress has been made over the past several years in our understanding of these issues and processes. Nevertheless, many questions remain about the detailed mechanisms of protection against poxviruses and the functions and maintenance of different compartments of immune memory. Will

the patterns of long-term maintenance of memory B cells, memory T cells, and antibody levels observed in these new studies hold true for situations other than smallpox immunization? Or do different immunizations/infections result in memory responses that behave differently? This will almost certainly be the case for chronic and latent infections (due to the intermittent presence of antigen), but it may also be true for different types of vaccines and acute infections. Moreover, do these patterns hold true for all memory lymphocyte subsets? These and other questions await future studies.

References

1. Cummings JF, Polhemus ME, Hawkes C, Klote M, Ludwig GV, Wortmann G. Lack of vaccinia viremia after smallpox vaccination. Clin Infect Dis 2004;**38**:456–458.
2. de Souza Trindade G, et al. Aracatuba virus: a vaccinialike virus associated with infection in humans and cattle. Emerg Infect Dis 2003;**9**:155–160.
3. Hassan A, Abdel Wahab MF, Farid Z, Imam ZE, Kent DC, Labib A. An outbreak of vaccinia in thirteen Egyptian slaughterhouse labourers at Cairo. J Trop Med Hyg 1970;**73**:51–53.
4. Nagasse-Sugahara TK, et al. Human vaccinia-like virus outbreaks in Sao Paulo and Goias States, Brazil: virus detection, isolation and identification. Rev Inst Med Trop Sao Paulo 2004;**46**:315–322.
5. Slifka MK. Immunological memory to viral infection. Curr Opin Immunol 2004;**16**:443–450.
6. Jenner E. An Inquiry into the Causes and Effects of the Variolae Vaccinae. London: Sampson Low, 1798.
7. Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. Smallpox and its Eradication. Geneva: World Health Organization, 1988.
8. Tscharke DC, Reading PC, Smith GL. Dermal infection with vaccinia virus reveals roles for virus proteins not seen using other inoculation routes. J Gen Virol 2002;**83**:1977–1986.
9. Wyatt LS, Earl PL, Eller LA, Moss B. Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. Proc Natl Acad Sci USA 2004;**101**:4590–4595.
10. Xu R, Johnson AJ, Liggitt D, Bevan MJ. Cellular and humoral immunity against vaccinia virus infection of mice. J Immunol 2004;**172**:6265–6271.
11. Davies DH, et al. Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. J Virol 2005;**79**:11724–11733.
12. Williamson JD, Reith RW, Jeffrey LJ, Arrand JR, Mackett M. Biological characterization of recombinant vaccinia viruses in mice infected by the respiratory route. J Gen Virol 1990;**71**:2761–2767.
13. Alcami A, Smith GL. A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. Cell 1992;**71**:153–167.
14. Belyakov IM, et al. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. Proc Natl Acad Sci USA 2003;**100**: 9458–9463.
15. Spriggs MK, et al. Beta 2-microglobulin-, CD8+ T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. Proc Natl Acad Sci USA 1992;**89**:6070–6074.
16. Coulibaly S, Bruhl P, Mayrhofer J, Schmid K, Gerencer M, Falkner FG. The nonreplicating smallpox candidate vaccines defective vaccinia Lister (dVV-L) and modified vaccinia Ankara (MVA) elicit robust long-term protection. Virology 2005;**341**:91–101.
17. Harrington LE, Most Rv R, Whitton JL, Ahmed R. Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. J Virol 2002;**76**: 3329–3337.
18. Brown DM, Roman E, Swain SL. CD4 T cell responses to influenza infection. Semin Immunol 2004;**16**:171–177.
19. Fuller MJ, Khanolkar A, Tebo AE, Zajac AJ. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. J Immunol 2004;**172**:4204–4214.
20. La Gruta NL, Turner SJ, Doherty PC. Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8+ T cell responses: correlation of cytokine profile and TCR avidity. J Immunol 2004;**172**:5553–5560.
21. Roman E, et al. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. J Exp Med 2002;**196**:957–968.
22. Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol 2003;**4**:835–842.
23. Carroll MW, Moss B. Poxviruses as expression vectors. Curr Opin Biotechnol 1997;**8**:573–577.
24. Franchini G, Gurunathan S, Baglyos L, Plotkin S, Tartaglia J. Poxvirus-based vaccine candidates for HIV: two decades of experience with special emphasis on canarypox vectors. Expert Rev Vaccines 2004;**3**:S75–S88.
25. Sandhu D, Tartaglia J. Poxviruses as immunization vehicles. In: Plotkin S, Orenstein W, eds. Vaccines, 4th edn. Saunders: Philadelphia, 2004:1297–1316.
26. Earl PL, et al. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature 2004;**428**:182–185.
27. Edghill-Smith Y, et al. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat Med 2005;**11**:740–747.
28. Law M, Putz MM, Smith GL. An investigation of the therapeutic value of vaccinia-immune IgG in a mouse pneumonia model. J Gen Virol 2005;**86**:991–1000.
29. Viner KM, Isaacs SN. Activity of vaccinia virus-neutralizing antibody in the sera of smallpox vaccinees. Microbes Infect 2005;**7**:579–583.
30. Davies DH, et al. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. Proc Natl Acad Sci USA 2005;**102**:547–552.
31. Bell E, Shamim M, Whitbeck JC, Sfyroera G, Lambris JD, Isaacs SN. Antibodies against the extracellular enveloped virus B5R protein are mainly responsible for the EEV neutralizing capacity of vaccinia immune globulin. Virology 2004;**325**:425–431.
32. Jones-Trower A, et al. Identification and preliminary characterization of vaccinia virus (Dryvax) antigens recognized by vaccinia immune globulin. Virology 2005;**343**:128–140.
33. McCarthy K, Downie AW, Bradley WH. The antibody response in man following infection with viruses of the pox group. II. Antibody response following vaccination. J Hyg (Lond) 1958;**56**:466–478.
34. Frey SE, Newman FK, Yan L, Lottenbach KR, Belshe RB. Response to smallpox vaccine in persons immunized in the distant past. JAMA 2003;**289**:3295–3299.

35. McClain DJ, et al. Immunologic responses to vaccinia vaccines administered by different parenteral routes. J Infect Dis 1997;**175**: 756–763.
36. Greenberg RN, et al. Safety and immunogenicity of new cell-cultured smallpox vaccine compared with calf-lymph derived vaccine: a blind, single-centre, randomised controlled trial. Lancet 2005;**365**:398–409.
37. Crotty S, Ahmed R. Immunological memory in humans. Semin Immunol 2004;**16**: 197–203.
38. Hammarlund E, et al. Duration of antiviral immunity after smallpox vaccination. Nat Med 2003;**9**:1131–1137.
39. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. J Immunol 2003;**171**:4969–4973.
40. el-Ad B, et al. The persistence of neutralizing antibodies after revaccination against smallpox. J Infect Dis 1990;**161**:446–448.
41. Manischewitz J, et al. Development of a novel vaccinia-neutralization assay based on reporter-gene expression. J Infect Dis 2003;**188**:440–448.
42. Putz MM, Alberini I, Midgley CM, Manini I, Montomoli E, Smith GL. Prevalence of antibodies to vaccinia virus after smallpox vaccination in Italy. J Gen Virol 2005;**86**: 2955–2960.
43. Demkowicz WE Jr, Ennis FA. Vaccinia virus-specific CD8+ cytotoxic T lymphocytes in humans. J Virol 1993;**67**:1538–1544.
44. Littaua RA, Takeda A, Cruz J, Ennis FA. Vaccinia virus-specific human CD4+ cytotoxic T-lymphocyte clones. J Virol 1992;**66**:2274–2280.
45. Amara RR, Nigam P, Sharma S, Liu J, Bostik V. Long-lived poxvirus immunity, robust CD4 help, and better persistence of CD4 than CD8 T cells. J Virol 2004;**78**:3811–3816.
46. Terajima M, et al. Quantitation of CD8+ T cell responses to newly identified HLA-A*0201-restricted T cell epitopes conserved among vaccinia and variola (smallpox) viruses. J Exp Med 2003;**197**:927–932.
47. Rock MT, Yoder SM, Wright PF, Talbot TR, Edwards KM, Crowe JE Jr. Differential regulation of granzyme and perforin in effector and memory T cells following smallpox immunization. J Immunol 2005;**174**:3757–3764.
48. Arita I. Duration of immunity after smallpox vaccination: a study on vaccination policy against smallpox bioterrorism in Japan. Jpn J Infect Dis 2002;**55**:112–116.
49. Mack TM. Smallpox in Europe, 1950–1971. J Infect Dis 1972;**125**:161–169.
50. Demkowicz WE, Maa JS, Esteban M. Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. J Virol 1992;**66**:386–398.
51. Wilton S, Gordon J, Dales S. Identification of antigenic determinants by polyclonal and hybridoma antibodies induced during the course of infection by vaccinia virus. Virology 1986;**148**:84–96.
52. Moss B. Poxviridae: The viruses and their replication. In: Knipe DM, Howley P, eds. Fundamental Virology, 4th edn. Philadelphia: Lippincott Williams & Wilkins, 2001.
53. Roos N, Cyrklaff M, Cudmore S, Blasco R, Krijnse-Locker J, Griffiths G. A novel immunogold cryoelectron microscopic approach to investigate the structure of the intracellular and extracellular forms of vaccinia virus. EMBO J 1996;**15**:2343–2355.
54. Smith GL, Vanderplasschen A, Law M. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol 2002;**83**:2915–2931.
55. Payne LG. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol 1980;**50**:89–100.
56. Lustig S, Fogg C, Whitbeck JC, Eisenberg RJ, Cohen GH, Moss B. Combinations of polyclonal or monoclonal antibodies to proteins of the outer membranes of the two infectious forms of vaccinia virus protect mice against a lethal respiratory challenge. J Virol 2005;**79**:13454–13462.
57. Galmiche MC, Goenaga J, Wittek R, Rindisbacher L. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. Virology 1999;**254**:71–80.
58. Ramirez JC, Tapia E, Esteban M. Administration to mice of a monoclonal antibody that neutralizes the intracellular mature virus form of vaccinia virus limits virus replication efficiently under prophylactic and therapeutic conditions. J Gen Virol 2002;**83**:1059–1067.
59. Ichihashi Y, Oie M. Neutralizing epitope on penetration protein of vaccinia virus. Virology 1996;**220**:491–494.
60. Ichihashi Y, Takahashi T, Oie M. Identification of a vaccinia virus penetration protein. Virology 1994;**202**:834–843.
61. Wolffe EJ, Vijaya S, Moss B. A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. Virology 1995;**211**:53–63.
62. Hsiao JC, Chung CS, Chang W. Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. J Virol 1999;**73**:8750–8761.
63. Rodriguez JF, Esteban M. Mapping and nucleotide sequence of the vaccinia virus gene that encodes a 14-kilodalton fusion protein. J Virol 1987;**61**:3550–3554.
64. Rodriguez JF, Janeczko R, Esteban M. Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. J Virol 1985;**56**:482–488.
65. Lai CF, Gong SC, Esteban M. The purified 14-kilodalton envelope protein of vaccinia virus produced in Escherichia coli induces virus immunity in animals. J Virol 1991;**65**:5631–5635.
66. Wallengren K, Risco C, Krijnse-Locker J, Esteban M, Rodriguez D. The A17L gene product of vaccinia virus is exposed on the surface of IMV. Virology 2001;**290**: 143–152.
67. Chertov O, et al. Amino acid sequence determination of vaccinia virus immunodominant protein p35 and identification of the gene. Biomed Sci 1991;**2**:151–154.
68. Lin CL, Chung CS, Heine HG, Chang W. Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. J Virol 2000;**74**:3353–3365.
69. Aldaz-Carroll L, et al. Epitope-mapping studies define two major neutralization sites on the vaccinia virus extracellular enveloped virus glycoprotein B5R. J Virol 2005;**79**:6260–6271.
70. Aldaz-Carroll L, et al. Physical and immunological characterization of a recombinant secreted form of the membrane protein encoded by the vaccinia virus L1R gene. Virology 2005;**341**:59–71.
71. Lustig S, Fogg C, Whitbeck JC, Moss B. Synergistic neutralizing activities of antibodies to outer membrane proteins of the two infectious forms of vaccinia virus in the presence of complement. Virology 2004;**328**:30–35.
72. Burton DR. Antibodies, viruses and vaccines. Nat Rev Immunol 2002;**2**:706–713.
73. Mack TM, Noble J Jr, Thomas DB. A prospective study of serum antibody and protection against smallpox. Am J Trop Med Hyg 1972;**21**:214–218.
74. Sarkar JK, Mitra AC, Mukherjee MK. The minimum protective level of antibodies in smallpox. Bull World Health Organ 1975;**52**:307–311.
75. Consortium PBR www.POXVIRUS.org. Poxvirus Bioinformatics Resource Center: Poxvirus Bioinformatics Resource Consortium, 2005.
76. Boulanger D, et al. Identification and characterization of three immunodominant structural proteins of fowlpox virus. J Virol 2002;**76**:9844–9855.

77. Heine HG, Stevens MP, Foord AJ, Boyle DB. A capripoxvirus detection PCR and antibody ELISA based on the major antigen P32, the homolog of the vaccinia virus H3L gene. J Immunol Methods 1999;**227**:187–196.
78. Housawi FM, et al. The reactivity of monoclonal antibodies against orf virus with other parapoxviruses and the identification of a 39 kDa immunodominant protein. Arch Virol 1998;**143**:2289–2303.
79. Mathew A, et al. Identification of murine poxvirus-specific CD8+ CTL epitopes with distinct functional profiles. J Immunol 2005;**174**:2212–2219.
80. Crowe SR, Miller SC, Shenyo RM, Woodland DL. Vaccination with an acidic polymerase epitope of influenza virus elicits a potent antiviral T cell response but delayed clearance of an influenza virus challenge. J Immunol 2005;**174**:696–701.
81. Belz GT, Xie W, Doherty PC. Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. J Immunol 2001;**166**:4627–4633.
82. Rodriguez F, Slifka MK, Harkins S, Whitton JL. Two overlapping subdominant epitopes identified by DNA immunization induce protective CD8(+) T-cell populations with differing cytolytic activities. J Virol 2001;**75**:7399–7409.
83. Wherry EJ, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol 2003;**4**:225–234.
84. Tscharke DC, et al. Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. J Exp Med 2005;**201**:95–104.
85. Robinson J, et al. IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. Nucleic Acids Res 2003;**31**:311–314.
86. Pasquetto V, et al. HLA-A*0201, HLA-A*1101, and HLA-B*0702 transgenic mice recognize numerous poxvirus determinants from a wide variety of viral gene products. J Immunol 2005;**175**:5504–5515.
87. Snyder JT, Belyakov IM, Dzutsev A, Lemonnier F, Berzofsky JA. Protection against lethal vaccinia virus challenge in HLA-A2 transgenic mice by immunization with a single CD8+ T-cell peptide epitope of vaccinia and variola viruses. J Virol 2004;**78**:7052–7060.
88. Drexler I, et al. Identification of vaccinia virus epitope-specific HLA-A*0201-restricted T cells and comparative analysis of smallpox vaccines. Proc Natl Acad Sci USA 2003;**100**:217–222.
89. Pinilla C, et al. Exploring immunological specificity using synthetic peptide combinatorial libraries. Curr Opin Immunol 1999;**11**:193–202.
90. Oseroff C, et al. HLA class I-restricted responses to vaccinia recognize a broad array of proteins mainly involved in virulence and viral gene regulation. Proc Natl Acad Sci USA 2005;**102**:13980–13985.
91. Shearer JD, Siemann L, Gerkovich M, House RV. Biological activity of an intravenous preparation of human vaccinia immune globulin in mouse models of vaccinia virus infection. Antimicrob Agents Chemother 2005;**49**:2634–2641.
92. Fang M, Sigal LJ. Antibodies and CD8+ T cells are complementary and essential for natural resistance to a highly lethal cytopathic virus. J Immunol 2005;**175**:6829–6836.
93. Kempe CH. Studies on smallpox and complications of smallpox vaccination. Pediatrics 1960;**25**:176–189.
94. Kempe CH, Berge TO, England B. Hyperimmune vaccinial gamma globulin. Pediatrics 1956;**18**:177–188.
95. Marennikova SS. The use of hyperimmune antivaccinia gamma-globulin for the prevention and treatment of smallpox. Bull World Health Organ 1962;**27**:325–330.
96. Peirce ER, Melville FS, Downie AW, Duckworth MJ. Antivaccinial gamma-globulin in smallpox prophylaxis. Lancet 1958;**2**:635–638.
97. Mack JE. Ribavirin: an antiviral agent with promise. Pediatr Nurs 1988;**14**:220–221.
98. Hammarlund E, et al. Multiple diagnostic techniques identify previously vaccinated individuals with protective immunity against monkeypox. Nat Med 2005;**11**:1005–1011.
99. Couzi G, Kircher JP. Immunotherapie de la Variole. Bulletin de l'Institut d'hygiene Maroc 1941;**1**:59–68.
100. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature 1984;**312**: 548–551.
101. Coulie PG, Coutelier J-P, Uyttenhove C, Lambotte P, Van Snick J. In vivo suppression of T-dependent antibody responses by treatment with a monoclonal anti-L3T4 antibody. Eur J Immunol 1985;**15**:638–640.
102. Wofsy D, Mayes DC, Woodcock J, Seaman WE. Inhibition of humoral immunity in vivo by monoclonal antibody to L3T4: studies with soluble antigens in intact mice. J Immunol 1985;**135**:1698–1701.
103. Vieira P, Rajewsky K. Persistence of memory B cells in mice deprived of T cell help. Int Immunol 1990;**2**:487–494.
104. Banchereau J, et al. The CD40 antigen and its ligand. Annu Rev Immunol 1994;**12**:881–922.
105. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. Proc Natl Acad Sci USA 1992;**89**:6550–6554.
106. Whitmire JK, Slifka MK, Grewal IS, Flavell RA, Ahmed R. CD40 ligand-deficient mice generate a normal primary cytotoxic T- lymphocyte response but a defective humoral response to a viral infection. J Virol 1996;**70**:8375–8381.
107. Edghill-Smith Y, et al. Smallpox vaccine does not protect macaques with AIDS from a lethal monkeypox virus challenge. J Infect Dis 2005;**191**:372–381.
108. Bartlett JG. Smallpox vaccination and patients with human immunodeficiency virus infection or acquired immunodeficiency syndrome. Clin Infect Dis 2003;**36**:468–471.
109. Redfield RR, Wright DC, James WD, Jones TS, Brown C, Burke DS. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N Engl J Med 1987;**316**:673–676.
110. Zagury D. Anti-HIV cellular immunotherapy in AIDS. Lancet 1991;**338**:694–695.
111. Hebeis BJ, et al. Activation of virus-specific memory B cells in the absence of T cell help. J Exp Med 2004;**199**:593–602.
112. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. Nature 2003;**421**:282–287.
113. Naniche D, et al. Decrease in measles virus-specific CD4 T cell memory in vaccinated subjects. J Infect Dis 2004;**190**:1387–1395.
114. Thomas HI, Aird HC. Maintenance of high-avidity rubella-specific IgG antibody and titres in recent HIV seroconvertors and in patients progressing to the AIDS-related complex and AIDS. J Med Virol 1999;**58**:273–279.
115. Flo RW, Nilsen A, Voltersvik P, Haukenes G. Serum antibodies to viral pathogens and toxoplasma gondii in HIV-infected individuals. Apmis 1993;**101**:946–952.
116. Leclerc C, Sedlik C, Lo-Man R, Charlot B, Rojas M, Deriaud E. Stimulation of a memory B cell response does not require primed helper T cells. Eur J Immunol 1995;**25**:2533–2538.

117. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol 2002;**2**:251–262.
118. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. Annu Rev Immunol 2000;**18**:83–111.
119. Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol 2002;**2**:982–987.
120. Tough DF, Sprent J. Immunological memory. In: Paul WE, ed. Fundamental Immunology. Philadelphia: Lippincott Williams & Wilkins, 2003: 865–899.
121. Tanchot C, Lemonnier FA, Perarnau B, Freitas AA, Rocha B. Differential requirements for survival and proliferation of CD8 naive or memory T cells. Science 1997;**276**:2057–2062.
122. Swain SL, Hu H, Huston G. Class II-independent generation of CD4 memory T cells from effectors. Science 1999;**286**:1381–1383.
123. Murali-Krishna K, Lau LL, Sambhara S, Lemonnier F, Altman J, Ahmed R. Persistence of memory CD8 T cells in MHC class I-deficient mice. Science 1999;**286**:1377–1381.
124. Kassiotis G, Garcia S, Simpson E, Stockinger B. Impairment of immunological memory in the absence of MHC despite survival of memory T cells. Nat Immunol 2002;**3**:244–250.
125. Sprent J, Surh CD. T cell memory. Annu Rev Immunol 2002;**20**:551–579.
126. Sprent J, Surh CD. Cytokines and T cell homeostasis. Immunol Lett 2003;**85**:145–149.
127. Homann D, Teyton L, Oldstone MB. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat Med 2001;**7**:913–919.
128. Varga SM, Selin LK, Welsh RM. Independent regulation of lymphocytic choriomeningitis virus-specific T cell memory pools: relative stability of CD4 memory under conditions of CD8 memory T cell loss. J Immunol 2001;**166**:1554–1561.
129. Becker TC, et al. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. J Exp Med 2002;**195**:1541–1548.
130. Goldrath AW, et al. Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. J Exp Med 2002;**195**:1515–1522.
131. Kieper WC, et al. Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells. J Exp Med 2002;**195**:1533–1539.
132. Lenz DC, et al. IL-7 regulates basal homeostatic proliferation of antiviral CD4+T cell memory. Proc Natl Acad Sci USA 2004;**101**:9357–9362.
133. Tan JT, Ernst B, Kieper WC, LeRoy E, Sprent J, Surh CD. Interleukin (IL) -15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. J Exp Med 2002;**195**:1523–1532.
134. Becker TC, Coley SM, Wherry EJ, Ahmed R. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. J Immunol 2005;**174**:1269–1273.
135. Parretta E, Cassese G, Barba P, Santoni A, Guardiola J, Di Rosa F. CD8 cell division maintaining cytotoxic memory occurs predominantly in the bone marrow. J Immunol 2005;**174**:7654–7664.
136. Honjo T, Alt F, Neuberger MS. Molecular Biology of B Cells. Amsterdam: Elsevier, 2004.
137. Kosco-Vilbois MH. Are follicular dendritic cells really good for nothing? Nat Rev Immunol 2003;**3**:764–769.
138. Haberman AM, Shlomchik MJ. Reassessing the function of immune-complex retention by follicular dendritic cells. Nat Rev Immunol 2003;**3**:757–764.
139. Mandel TE, Phipps RP, Abbot A, Tew JG. The follicular dendritic cell: long term antigen retention during immunity. Immunol Rev 1980;**53**:29–59.
140. Bachmann MF, Zinkernagel RM. Neutralizing antiviral B cell responses. Annu Rev Immunol 1997;**15**:235–270.
141. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. Nature 2000;**407**:636–642.
142. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell 1997;**90**:1073–1083.
143. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;**298**:2199–2202.
144. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity 1998;**8**:363–372.
145. Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. J Virol 1995;**69**:1895–1902.
146. Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. Annu Rev Immunol 2005;**23**:367–386.
147. Tarlinton DM, Hodgkin PD. Targeting plasma cells in autoimmune diseases. J Exp Med 2004;**199**:1451–1454.
148. Ho F, Lortan JE, MacLennan IC, Khan M. Distinct short-lived and long-lived antibody-producing cell populations. Eur J Immunol 1986;**16**:1297–1301.
149. Makela O, Nossal GJ. Autoradiographic studies on the immune response. II. DNA synthesis amongst single antibody-producing cells. J Exp Med 1962;**115**:231–244.
150. Nossal GJ, Makela O. Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation. J Exp Med 1962;**115**:209–230.
151. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. Nature 1997;**388**:133–134.
152. Plotkin SA, Orenstein WA. Vaccines. Philadelphia: W.B. Saunders Co, 1999.
153. Leyendeckers H, et al. Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen- specific IgG. Eur J Immunol 1999;**29**:1406–1417.
154. Nanan R, Heinrich D, Frosch M, Kreth HW. Acute and long-term effects of booster immunisation on frequencies of antigen-specific memory B-lymphocytes. Vaccine 2001;**20**:498–504.
155. Cambridge G, et al. Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. Arthritis Rheum 2003;**48**:2146–2154.
156. Yeh WW, Moss B, Wolffe EJ. The vaccinia virus A9L gene encodes a membrane protein required for an early step in virion morphogenesis. J Virol 2000;**74**:9701–9711.
157. Unger B, Traktman P. Vaccinia virus morphogenesis: a13 phosphoprotein is required for assembly of mature virions. J Virol 2004;**78**:8885–8901.
158. Betakova T, Wolffe EJ, Moss B. The vaccinia virus A14.5L gene encodes a hydrophobic 53-amino-acid virion membrane protein that enhances virulence in mice and is conserved among vertebrate poxviruses. J Virol 2000;**74**:4085–4092.
159. Townsley AC, Senkevich TG, Moss B. Vaccinia virus A21 virion membrane protein is required for cell entry and fusion. J Virol 2005;**79**:9458–9469.
160. Senkevich TG, Ward BM, Moss B. Vaccinia virus entry into cells is dependent on a virion surface protein encoded by the A28L gene. J Virol 2004;**78**:2357–2366.
161. Szajner P, Weisberg AS, Lebowitz J, Heuser J, Moss B. External scaffold of spherical immature poxvirus particles is made of protein trimers, forming a honeycomb lattice. J Cell Biol 2005;**170**:971–981.
162. Sodeik B, Griffiths G, Ericsson M, Moss B, Doms RW. Assembly of vaccinia virus: effects of rifampin on the intracellular distribution of viral protein p65. J Virol 1994;**68**:1103–1114.

163. Heljasvaara R, Rodriguez D, Risco C, Carrascosa JL, Esteban M, Rodriguez JR. The major core protein P4a (A10L gene) of vaccinia virus is essential for correct assembly of viral DNA into the nucleoprotein complex to form immature viral particles. J Virol 2001;**75**:5778–5795.
164. Senkevich TG, Weisberg AS, Moss B. Vaccinia virus E10R protein is associated with the membranes of intracellular mature virions and has a role in morphogenesis. Virology 2000;**278**:244–252.
165. Su HP, Garman SC, Allison TJ, Fogg C, Moss B, Garboczi DN. The 1.51 Angstrom structure of the poxvirus L1 protein, a target of potent neutralizing antibodies. Proc Natl Acad Sci USA 2005;**102**:4240–4245.
166. Senkevich TG, Moss B. Vaccinia virus H2 protein is an essential component of a complex involved in virus entry and cell-cell fusion. J Virol 2005;**79**:4744–4754.
167. Takahashi T, Oie M, Ichihashi Y. N-terminal amino acid sequences of vaccinia virus structural proteins. Virology 1994;**202**:844–852.
168. Townsley AC, Senkevich TG, Moss B. The product of the vaccinia virus L5R gene is a fourth membrane protein encoded by all poxviruses that is required for cell entry and cell-cell fusion. J Virol 2005;**79**:10988–10998.
169. Frischknecht F, et al. Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. Nature 1999;**401**:926–929.
170. Rottger S, Frischknecht F, Reckmann I, Smith GL, Way M. Interactions between vaccinia virus IEV membrane proteins and their roles in IEV assembly and actin tail formation. J Virol 1999;**73**:2863–2875.
171. Isaacs SN, Wolffe EJ, Payne LG, Moss B. Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. J Virol 1992;**66**:7217–7224.
172. Engelstad M, Howard ST, Smith GL. A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. Virology 1992;**188**:801–810.
173. Condit RC, Moussatche N, Traktman P. In a nutshell: structure and assembly of the vaccinia virion. Advances in Virus Research; **66**: (in press).