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Germinal center enhancement by extended antigen availability

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Vaccine elicitation of protective antibody responses has proved difficult for a number of important human pathogens, including HIV-1. The amount of somatic hypermutation associated with the development of broadly neutralizing antibodies against HIV has not been achieved using conventional immunization strategies. An underexplored aspect of vaccine design is modulation of antigen kinetics. Immunization strategies with extended antigen availability have recently been shown to enhance humoral responses. In this review, we explore the mechanisms through which sustained antigen availability can enhance germinal center responses and the potency of antibody responses. These potential mechanisms include shifting B cell recognition away from non-neutralizing immunodominant epitopes, altered kinetics of immune complex deposition, improved T follicular helper (Tfh) cell responses, enhanced affinity maturation, and enhanced development of B cell memory. Finally, we discuss immunization strategies that result in extended antigen availability.

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Introduction

Most licensed human vaccines rely on antibody-mediated responses for protection. Those responses are primarily dependent on CD4⁺ T cells and germinal centers (GCs). GCs are sites within lymphoid organs where B cells undergo B cell receptor (BCR) somatic hypermutation (SHM) to enhance BCR affinity for antigen. Knowledge of the GC processes can almost certainly improve rational vaccine design, if parameters that modulate those

processes can be understood. The use of model protein antigens has provided considerable insight into the mechanisms underlying GC and antibody responses. However, the use of simple model antigens most likely does not reflect the immunological challenges presented by more complex pathogen antigens, which have potentially been driven by eons of evolution to be difficult for host B cells to recognize and neutralize. Few mutations are required for development of high-affinity antibodies against most simple model antigens, including the most commonly studied model antigen 4-hydroxy-3-nitrophenyl acetyl (NP), which only requires a single BCR amino acid mutation to develop high affinity antibodies [1]. Protective antibodies against some pathogens, including HIV-1, contain high numbers of amino acid mutations (>10) and develop over extended periods of time during infection [2,3]. Lastly, the lifespan of GCs elicited by model antigens can also be short compared to even acute natural infections, where there is frequently a prolonged supply of antigen and GC reactions can last many weeks [4]. Thus, experimental studies of more complex antigens are necessary to study the importance of GC parameters involved in the development of potent antibodies against difficult epitopes on pathogens [5].

One example of a difficult pathogen antigen for B cell recognition and neutralization is HIV envelope (Env). Approximately 10% of HIV⁺ individuals develop potent broadly neutralizing antibodies (bnAbs) targeting HIV Env [3]. These bnAbs take multiple years to develop and accumulate more amino acid mutations than antibodies generated during conventional immunizations. Many HIV bnAbs require rare SHM events, including deletions or combinatorial mutations (e.g. addition of a new disulfide bond across a CDR loop). Longitudinal analyses of BCR and viral lineages throughout HIV infection has provided clear evidence that bnAbs undergo high amounts of affinity maturation before obtaining their broadly neutralizing activity [6,7]. The development of bnAbs via immunization is a major challenge and it is likely that certain conditions that resemble natural infection, including persistent antigen presence, are required for HIV bnAb development [8].

A promising avenue in rational vaccine design for modulating GCs is the sustained delivery of antigen, which can more mimic natural infection. Sporadic studies more than a decade ago found that controlled release of antigen over a longer period of time could result in stronger immune responses than conventional bolus injections [9–11].

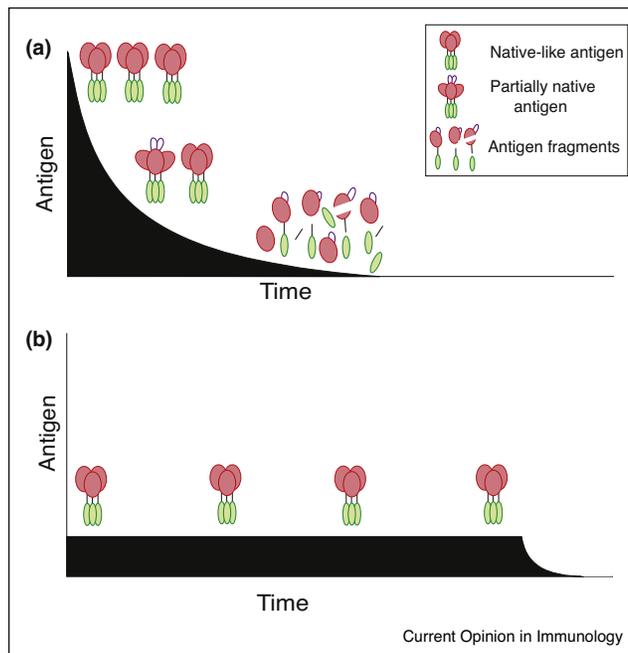
More recent studies have revisited this concept with substantial success [12^{**},13,14^{**}]. Here we describe several mechanisms through which sustained antigen availability may modulate the GC response to enhance the humoral response. These mechanisms include 1) increased availability of native antigen, 2) increased immune complex deposition, 3) modulation of Tfh help and affinity maturation, and 4) modulation of memory B cell formation. Lastly, we discuss the implications of these immunological processes and extended antigen strategies for vaccine design.

Availability of intact protein antigen

GC B cells with the highest affinity for antigen are selected to survive and proliferate based on the ability of the B cell to strip antigen from follicular dendritic cells (FDCs) and subsequently receive help from Tfh cells. One should consider how that process aligns with conventional immunizations. Conventional protein immunizations deliver antigen and adjuvant in a single bolus injection. A potential shortcoming of that strategy is that it is not synchronized with the GC response. The GC response peaks weeks after initial antigen exposure [15]. It is likely that the highest number of B cells are undergoing affinity maturation weeks after initial antigen exposure. It is important to consider that all proteins have a half-life and are susceptible to degradative processes over time. Thus, for proteins that do not exhibit exceptional stability, it is likely that at the peak of the GC response after a conventional immunization much of the antigen presented by FDCs to GC B cells is nonnative protein and protein degradation products, which expose epitopes that are normally hidden or nonexistent on the native form of the protein (Figure 1). That is a potentially problematic and counterproductive situation. There are data suggesting that in some cases, nonnative epitopes can be immunodominant and distract the GC response from relevant targets.

HIV Env is one such example. Env is the only target of HIV-neutralizing antibodies. The protein is made up of three gp120 and three gp41 molecules. Immunization with gp120 or labile Env fails to elicit neutralizing antibodies against most circulating HIV strains (frequently called Tier 2 and 3 viruses) [16,17]. Most antibodies from these immunizations are directed against the V3 loop, which is normally hidden within the intact trimer, and thus such antibodies are largely irrelevant. Disorganization or degradation of the Env trimer can result in the exposure of the V3 loop and other non-neutralizing epitopes, which could potentially be immunodominant over neutralizing epitopes. We recently demonstrated proof of concept of antigen integrity impacting GC responses and B cell epitope dominance. The response against the V3 loop can be drastically reduced in mice by sequestering the Env trimer and adjuvant in osmotic minipumps [13^{*}]. Osmotic minipumps are nonmechanical delivery systems

Figure 1



Slow immunogen release improves the availability of intact antigen.

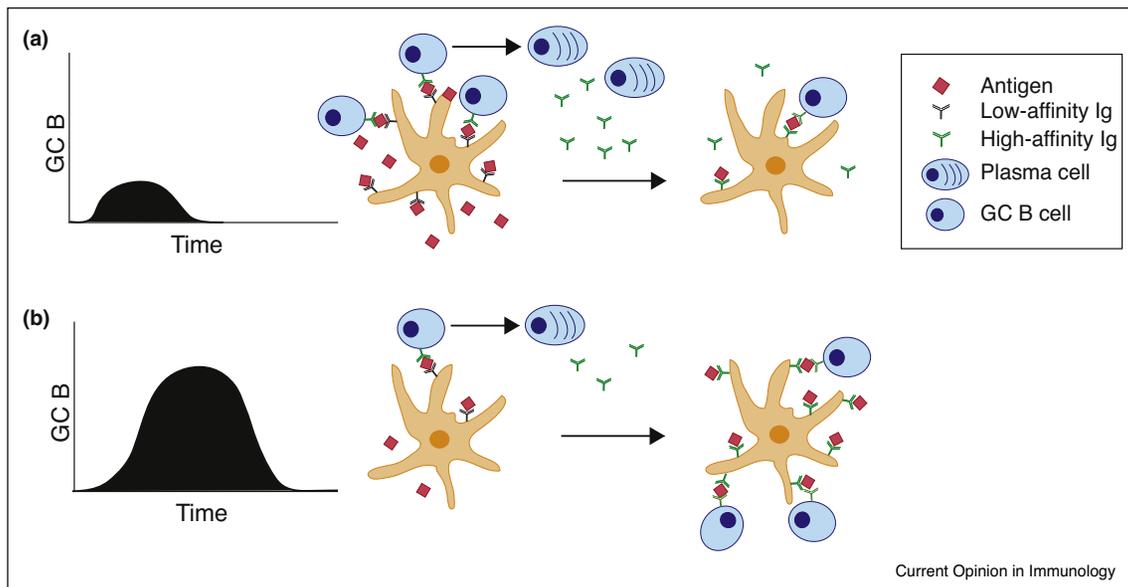
(a) Soluble immunogen can lose native structure due to lability or proteolysis over time. This can lead to exposure of irrelevant and potentially distracting epitopes. (b) Sustained release of immunogen limits the amount of soluble antigen available to proteases, and ensures availability of intact immunogen at later times during the immune response.

implanted subcutaneously to continuously supply material at a fixed rate over several days or weeks. Each immunization of immunogen plus adjuvant was slowly delivered over one or two weeks. After three immunizations, the antibody response of conventional bolus-immunized mice was frequently dominated by the V3-response. In contrast, the V3-response was no longer dominant in osmotic pump-immunized mice. Sustained release of native trimers was sufficient to shift B cell recognition towards more native epitopes. Naïve B cells that target neutralizing epitopes are likely immunorecessive for difficult to neutralize pathogens (i.e. rare and/or of very low affinity compared to immunodominant epitope targets). The likelihood that rare B cells with neutralizing specificity will be successfully recruited increases when most, if not all, antigen in the immunization maintains its native form. Sustained antigen delivery is therefore an attractive strategy in vaccine design for labile antigens, or antigens for which the B cells specific to critical epitopes are known or suspected of being immunorecessive.

Immune complex deposition

FDCs concentrate and present intact antigen in the form of immune complexes to B cells in the light zone (LZ) of the GC [18,19]. B cells test their evolving affinity on FDC

Figure 2



Sustained immunogen release enhances immune complex deposition and GCs. Low affinity antibodies bind antigen as immune complexes and are deposited on follicular dendritic cells (FDCs). Some B cells that have sufficient affinity to antigen will differentiate into plasma cells early in the immune response and secrete higher affinity antibodies, which then bind remaining free antigen. **(a)** During a conventional protein immunization, most antigen is presented to B cells early relative to the peak of the GC response. Thus, B cell affinity maturation rates may peak relatively early under those conventional immunization conditions. **(b)** Sustained antigen release immunization introduces new antigen during a time window when higher affinity IgG is produced, allowing for increased immune complex formation and deposition onto FDCs. This antigen is retained at higher concentrations and for longer durations on FDCs. Thus, the magnitude of the GC response is larger, and B cell affinity maturation rates may be sustained better than under conventional immunization conditions.

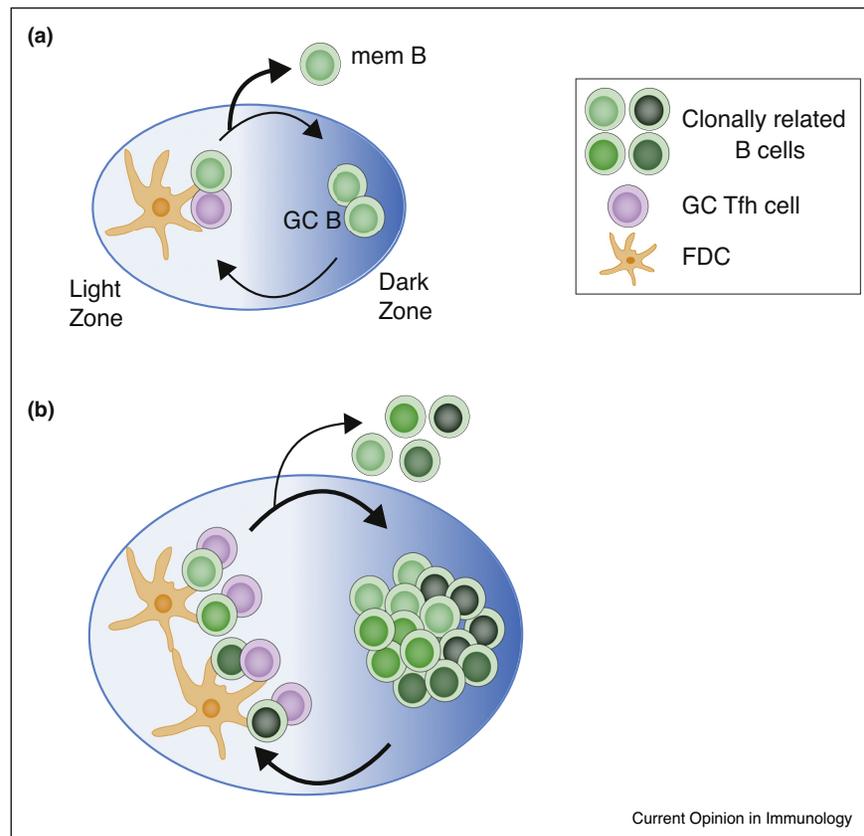
immune complexes. B cells with BCRs with higher affinity to the antigen are selected to survive and undergo an additional round of affinity maturation or develop into plasma cells. The resulting higher affinity IgG from newly generated plasma cells can then form new immune complexes, replacing the lower affinity immune complexes in a recursive process. This results in longer retention of antigen on FDCs (Figure 2). If concentrations of high affinity antibody become sufficiently high, all antigen becomes complexed with antibody. This can lead to GC termination [20,21]. The effects of extended antigen availability immunization strategies on antigen retention by FDCs and the antibody response were recently explored [12^{••}]. Mice that were given repeated, exponentially increasing immunization doses retained antigen in their draining LNs significantly longer than mice given a single bolus. This was correlated with improved immune complex formation on FDCs. Immunogen was provided during a window of time when antigen-specific antibody was available for immune complex formation (>d6 post-immunization). This shift in immune complex kinetics is significant, as the peak of the GC response can frequently occur multiple weeks after an immunization. More antigen on FDCs during later stages of a GC likely increases GC B cell clonotypic diversity (Figure 2), allowing for significantly more BCR sequence space to be explored for

high affinity BCR mutations. During a conventional immunization, most antigen presentation, and thus B cell selection, likely occurs before the peak GC response. Interestingly, administration of the same dosing profile over different time frames resulted in differences in the humoral response. Immunization over one or three weeks resulted in significantly lower antibody titers compared to immunization over two weeks [12^{••},13[•]]. Altogether, these data indicate that extended antigen availability improves immune complex formation and thus improves the magnitude and quality of GCs.

Tfh cell help and affinity maturation

T follicular helper (Tfh) cells are required for GC development and function [22]. GC B cells with the highest affinity capture and present the most antigen, in the form of peptide-MHCII complexes to GC Tfh cells in the LZ. GC Tfh cells selectively help B cells that present the most antigen by providing signals essential for GC B cell survival, proliferation, and mutation [23,24]. Surviving GC B cells migrate to the dark zone (DZ), where they proliferate and undergo SHM. Mutated GC B cells migrate back to the LZ, where the highest affinity B cells are selected again by GC Tfh cells. The amount of help provided by GC Tfh to GC B cells determines the rate at which B cells proliferate and the number of

Figure 3



A model of Tfh help modulation by extended immunogen release. **(a)** During a conventional immunization, the presence of low numbers of GC Tfh cells usually restricts the number of GC B cells, limiting BCR diversity. **(b)** Extended immunogen release can support increased GC Tfh cell numbers, or greater GC Tfh help quality. More GC B cells are able to proliferate and undergo SHM in the presence of increased GC Tfh cell help. More GC Tfh help both generates a larger pool of clonotypic BCR diversity and lowers competition between B cells, allowing for the generation of GC B cells with more complex BCR antigen recognition features.

mutations the B cell acquires within a given selection cycle [24,25^{••}]. GC Tfh cells therefore control the amount of clonotypic BCR diversification within a GC. High affinity antibodies against simple antigens require only a few mutations. Thus, the BCR diversity pool (i.e. the number of GC B cells) necessary to develop these antibodies is relatively small. Hence, stringent Tfh help, in the form of limited numbers of GC Tfh cells, is likely optimal for affinity maturation to simple antigens. In contrast, to develop high affinity antibodies against more complex antigens, high rates of SHM are likely required. Therefore, higher numbers of GC Tfh cells or greater GC Tfh help is needed in GC responses to complex antigens to maintain a larger pool of diverse BCRs and increase the possibility of developing high affinity nAbs (Figure 3). Lastly, strong GC Tfh help is likely essential for the development of some bnAbs that specifically require rare mutation events like the addition of disulfide bonds [26]. Generation of two cysteine mutations within a given GC cycle may be necessary as a single cysteine mutation is likely detrimental to BCR affinity. With strong GC Tfh

help, GC B cells can accumulate multiple mutations in a single round of SHM. Thus, enhancement of the Tfh cell response is likely of value in the context of immunization against antigens that are difficult for B cell to recognize.

On the basis of the basic biology of CD4⁺ T cells, and Tfh cells in particular [27,28], it was likely that immunization strategies utilizing sustained antigen delivery would increase the abundance of Tfh cells. Indeed, continuous antigen release over 1–2 weeks using osmotic pumps in mice resulted in higher frequencies of GC Tfh, Tfh and GC B cells [12^{••}]. Strikingly, non-human primates (NHPs) immunized with HIV Env via two week osmotic pumps had dramatically increased autologous HIV nAbs compared to animals given a conventional bolus immunization [14^{••}]. Furthermore, HIV nAbs also developed in osmotic pump immunized animals more rapidly than conventional animals, suggesting that extended antigen release can accelerate the generation of GC B cells capable of neutralizing HIV. Osmotic pump immunized NHPs had significantly higher frequencies of GC Tfh

cells. Interestingly, higher frequencies of Ki67⁺ GC Tfh were observed in pump animals compared to the conventionally immunized animals. Altogether, these data demonstrate that sustained antigen administration alters the GC response and can have an impressive impact on the resulting antibody response. Whether an increase in GC Tfh cell frequency results in increased rates of GC B cell SHM remains to be determined. Overall, it is now of great interest to directly investigate the effect of extended antigen availability on Tfh cell biology and the Tfh-dependent processes of SHM and affinity maturation against difficult antigens.

Development of memory

Vaccination strategies to elicit memory B cells (MBCs) that can reenter GCs are attractive, as it is highly unlikely a single conventional immunization will allow for the extensive mutations required for neutralization of some pathogens. Few, if any, studies have explored the effect of vaccination with persistent antigen on the development of memory. Antigen kinetics may affect the pathways that control the decisions to differentiate into MBCs or PCs. B cells with lower affinity preferentially become MBCs early relative to the peak of the GC response [29[•],30]. Immunizations with sustained antigen may delay MBC development relative to a conventional bolus. This may allow GC B cells to accumulate more mutations, and thus higher affinity, before exiting GCs as MBCs. There is currently no evidence to support or refute this hypothesis, but insights into these processes may provide considerable guidance in vaccine design.

Delivery systems and adjuvants

Osmotic pumps have been used for sustained immunization delivery in several proof-of-principle animal studies. While surgical implantation of osmotic pumps may be feasible for a Phase I human vaccine trial, osmotic pumps are clearly impractical for large scale human vaccine trials. Near daily repeated injections are also impractical for large scale human vaccine trials. More pragmatic delivery systems include slow release microneedles implanted through the skin with a patch and injectable microparticles [31[•],32,33]. Additionally, it has long been believed that some adjuvants, most notably alum, modulate humoral immune responses via a ‘depot effect’, in which antigen is retained at the site of injection and slowly released [34,35]. However, the adjuvanticity of alum is not dependent on depot formation. Surgical removal of alum depots two hours after injection did not affect the magnitude of the antibody response in mice [36]. Nevertheless, it remains unknown how much of the effect of other adjuvants on GCs is due to extended antigen release. The formation of an adjuvant-antigen depot in and of itself is not informative, as it is not clear that the antigen trapped in the depot becomes available to lymphocytes at later times. The duration and kinetics of antigen release are unknown for many adjuvants. Thus,

these recent advances demonstrating the value of extended antigen release highlight that a mechanistic understanding of the depot effects of adjuvants is needed. Such experiments may need to be done with difficult or immunorecessive antigens to accurately assess their impact on Tfh cells, GCs, and humoral immunity, for the reasons described above.

Summary

Protein vaccines safely elicit protective responses in humans and have greatly improved public health globally. For some pathogens with difficult neutralizing epitopes, nontraditional vaccination strategies may be required for long-lived immunity. It will be important to further understand how modulating immunogen kinetics impacts Tfh cell help, SHM, and MBC formation. Insights into the kinetics of the GC response, particularly in humans and NHPs, will benefit the efforts to optimize vaccination against difficult targets.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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