HYPOTHESIS
Insights & Perspectives

Physical ‘strength’ of the multi-protein chain connecting immune cells: Does the weakest link limit antibody affinity maturation?

The weakest link in the multi-protein chain facilitating antigen acquisition by B cells in germinal centres limits antibody affinity maturation.

Rajat Desikan1 | Rustom Antia2 | Narendra M. Dixit1,3

1 Department of Chemical Engineering, Indian Institute of Science, Bengaluru, India
2 Department of Biology, Emory University, Atlanta, Georgia, USA
3 Centre for Biosystems Science and Engineering, Indian Institute of Science, Bengaluru, India

Abstract
The affinities of antibodies (Abs) for their target antigens (Ags) gradually increase in vivo following an infection or vaccination, but reach saturation at values well below those realisable in vitro. This ‘affinity ceiling’ could in many cases restrict our ability to fight infections and compromise vaccines. What determines the affinity ceiling has been an unresolved question for decades. Here, we argue that it arises from the strength of the chain of protein complexes that is pulled by B cells during the process of Ag acquisition. The affinity ceiling is determined by the strength of the weakest link in the chain. We identify the weakest link and show that the resulting affinity ceiling can explain the Ab affinities realized in vivo, providing a conceptual understanding of Ab affinity maturation. We explore plausible evolutionary underpinnings of the affinity ceiling, examine supporting evidence and alternative hypotheses and discuss implications for vaccination strategies.

KEYWORDS
affinity ceiling, antibody affinity maturation, B cell, Bell model, evolution, follicular dendritic cell, germinal centre, humoral immunity, rupture force, vaccine

INTRODUCTION

Antibody affinities hit a ceiling in vivo

Antibodies (Abs) produced later in an infection tend to have higher affinities for their target antigen (Ag) than those produced earlier.[1,2] This phenomenon, termed Ab affinity maturation (AM),[1,2] is a hallmark of humoral immunity and demonstrates its ability to ‘learn’ to recognize its target better with time. When challenged with simple Ags such as haptens conjugated to proteins, mice produced Ag-specific Abs with affinities, quantified using the equilibrium association constant ($K_A$) that increased from $10^5$–$10^6$ M$^{-1}$ a week after challenge to $10^7$–$10^8$ M$^{-1}$ in a few months.[1] A similar rise is seen for complex Ags: the affinity of broadly neutralising antibodies (bNAbs) of HIV-1, which target conserved regions on the HIV-1 envelope, rose gradually from $\sim 10^4$ M$^{-1}$ to $10^8$–$10^9$ M$^{-1}$ in a few years in HIV-1 infected individuals (Figure 1A).[4,5] The mean affinity of Abs against hemagglutinin during influenza infection was similarly found to rise by up to 1000-fold.[6,7] Ab affinities can thus rise many orders of magnitude during the course of an infection.

This rise, however, is not unabated. The affinity eventually reaches a ceiling. The maximum affinities realized in vivo correspond to $K_A$ of $\sim 10^{10}$–$10^{12}$ M$^{-1}$ (Figure 1B).[11–13] Intriguingly, this saturating affinity is far below the maximum affinity realisable between proteins.
FIGURE 1  The antibody affinity ceiling. (A) Affinity maturation of the CH103 anti-HIV-1 broadly neutralising antibody lineage towards heterologous HIV antigen over years (from 14 weeks to 136 weeks) of infection.[4] The horizontal black line indicates the mean $K_A$ of the last three members of the lineage. Members are indicated on the x-axis. (B) Histogram of Ab–Ag affinities reported in the structural antibody database (SAbDAb).[8] Vertical black- and red-dashed lines correspond to $K_A$ of $2.1 \times 10^{13}$ M$^{-1}$ and $10^{15}$ M$^{-1}$, respectively, representing the highest Ab–Ag affinity in vitro[9] and the highest protein–protein affinity reported,[10] illustrating the gap between the affinities realisable and those realized in vivo, limited by the affinity ceiling.

or Ab–Ag pairs. The maximum $K_A$ recorded between proteins, which is for the avidin-biotin interaction, is $\sim 10^{15}$ M$^{-1}$. Abs with $K_A > 10^{13}$ M$^{-1}$ for their target Ags have been realized in vitro using directed evolution.[9] AM in vivo thus appears to hit an ‘affinity ceiling’.[11,12] The potency with which Abs neutralize their targets is positively correlated with their affinities for the targets.[14–16] The ceiling thus potentially determines the strength of the endogenous Ab response. Unravelling the origins of the affinity ceiling and devising ways to manipulate it would have implications not only for our understanding of the Ab response, but also for optimising vaccinations and immunotherapies for infections and cancer.[17,18]

Ab AM occurs in germinal centres via localized Darwinian evolution of B cells

AM occurs in germinal centres (GCs), which are temporary structures assembled in secondary lymphoid organs following an infection (Figure 2).[2,3,19] Here, B cells, termed GC B cells, continuously evolve and get selected based on the affinity of their B cell receptors (BCRs) for their target Ag. GCs are divided anatomically into a light zone and a dark zone (Figure 2). GC B cells are pro-apoptotic by default and must receive two signals in the light zone to survive.[20] The first signal is from Ag bound to BCR on Ag-specific B cells. This results in the phagocytosis of the Ag, which is processed and presented to T follicular helper ($T_{fh}$) cells, which deliver ‘help’, the second signal, to the B cells. When these two signals are received, the B cells are rescued. A majority then migrate to the dark zone, where they proliferate and mutate their BCR, or Ab, genes. Abs are secreted forms of BCRs; the two have identical Ag binding regions or ‘paratopes’. They then migrate back to the light zone, where they are subjected to the same survival pressures again. The likelihood of the receipt of survival signals depends on the affinity of the BCR for Ag. Each B cell expresses a single kind of BCR.

The greater the affinity, the greater is the chance of Ag acquisition and hence also of receiving $T_{fh}$ cell help. Consequently, progressively, B cells with increasing affinity for the target Ag are selected, resulting in AM. A small fraction of selected GC B cells differentiates into plasma cells, exits the GC and produces Abs (Figure 2), explaining the observed increase in the affinity of the Abs with time.

The prevalent paradigm: constraints on ‘on’ and ‘off’ rates determine the affinity ceiling (Foote and Eisen model)

Why does AM saturate? In an insightful commentary nearly 25 years ago, Foote and Eisen answered this question for the scenario wherein B cells acquire soluble Ag.[11] They argued that the affinity ceiling would arise from limits on the association and dissociation rate constants, $k_{on}$ and $k_{off}$, respectively, of the Ab–Ag interactions.[11] They reasoned that $k_{on}$ is limited by the diffusion of Abs to the soluble Ag, as has been verified experimentally and using simulations,[21,22] and could be set to a maximum of $10^5$–$10^6$ M$^{-1}$ s$^{-1}$. $k_{off}$, on the other hand, is not sensitive to the specific inter-molecular interactions, defined by the conformations, electrostatics, hydrophobicity and other interactions between the proteins. The difference in the affinity between different Abs for a given Ag must thus arise from different values of $k_{off}$. This difference is expected to be manifest in B cell selection. The smaller the $k_{off}$, the more stable would be the BCR–Ag complex on the B cell surface, a situation that could provide an advantage to the B cell in terms of longer stimulation of BCR signalling and/or higher probability of Ag uptake. Foote and Eisen recognized that this selective advantage would cease once $k_{off}$ approached the rate of internalisation of BCR–Ag complexes by B cells. All BCR–Ag complexes lasting longer (i.e., with smaller values of $k_{off}$) would be internalized and processed similarly, leaving little room for affinity discrimination. Thus, the internalisation, or
Recent insights challenge the prevalent paradigm

Recent experimental advances suggest that the process of Ag acquisition by B cells is more complex. Our view of the GC today differs fundamentally from the scenario on which the arguments of Foote and Eisen are predicated. In particular, advances in intravital imaging technologies, which allow direct visualisation of B cells within GCs, have established that B cells migrate towards follicular dendritic cells (FDCs) in GCs and acquire Ag not in soluble form but presented as Ab–Ag immune complexes (ICs) on the surfaces of the FDCs. The limits on $k_\text{on}$ and $k_\text{off}$, central to the affinity ceiling deduced by Foote and Eisen, may no longer apply. What then defines the ceiling?

Here, we suggest that the affinity ceiling arises from limits to the strength of the chain of protein complexes that is formed between GC B cells and FDCs for Ag acquisition, and that the limit is set by the weakest link in this chain.

**THE WEakest LINK HYpOTHESIS**

**Ag acquisition by B cells in GCs is facilitated by a chain of protein complexes**

We focussed on the process of Ag acquisition by B cells in GCs to examine how limits to AM could arise. We reasoned that akin to the arguments of Foote and Eisen, constraints extrinsic to Ab–Ag affinities may exist that limit AM. To elucidate these constraints, we considered a single Ag acquisition event orchestrated by a B cell through a single BCR (Figure 3A). Such an approach is justified by recent observations indicating that GC B cells express fewer BCRs and integrins and other anchoring proteins involved in synapse formation between B cells and antigen-presenting cells compared to naive B cells. Further, unlike naive B cells that spread over a surface presenting Ag and form uniform contacts, GC B cells form highly dynamic, punctate contacts, limiting the number of Ag–BCR complexes that can be formed.

We considered the scenario before Ag acquisition, where the B cell surface is apposed to the FDC surface and the BCR is complexed with its target Ag in the GC B cell–FDC contact region. The Ag is in a presented IC, the latter attached to either an FcγRIIB or CR2 receptor anchored to the FDC surface (Figure 3A). Ag is typically trafficked in the form of ICs by follicular B cells in lymph nodes (or marginal zone B cells in the spleen) using their complement receptors, in an Ag non-specific manner, to the FDC surface, where it is attached to FcγRIIB or CR2 receptors. The Abs forming the ICs are initially of low affinity (typically IgMs), but as AM proceeds, Abs (typically IgGs) with increasing affinity for the Ag replace the lower affinity Abs, a process recognized as Ab feedback (Figure 2), and increase the selection stringency in GCs. The BCR is subjected to a mechanical pulling force by the contractile motion of the B cell surface. This force is transmitted through the chain of protein complexes that links the B cell to the FDC. This chain involves the following complexes: BCR–Ag, Ag–Ab (IC) and Ab–FcyRIIB receptor (or Ab–CR2 receptor). In addition, the BCR is anchored to
The B cell–FDC tug-of-war, weakest link in the multi-protein chain, and affinity maturation regimes. (A) GC B cells interact with Ag-presenting FDCs in the light zone of the GC. The B cell–FDC contact (right zoom) is through a chain of non-covalently bound protein–protein and proteo-lipid complexes: FDC membrane with Fc\(\gamma\)RIIB/CR2 receptors; Fc\(\gamma\)RIIB/CR2 receptors with the presenting Ab in the IC; the presenting Ab with Ag; Ag with BCR; and BCR with the B cell membrane. The Ab/BCR interactions with the Ag occur at the respective epitopes (triangles). Energetically, the weakest link in this interacting chain upon sufficient Ab affinity maturation is the Fc\(\gamma\)RIIB/CR2–Ab complex. (B) (Left) Progressive affinity maturation. Ab–Ag interaction is the weakest link and Ag acquisition by B cells happens by BCRs breaking the Ab–Ag complex. B cells with higher affinity BCRs will thus acquire Ag more efficiently and typically end up with higher amounts of Ag than B cells with lower affinity BCRs. (Right) Saturating affinity maturation. Fc\(\gamma\)RIIB/CR2–Ab interaction is the weakest link, and B cells acquire Ag by breaking the Fc\(\gamma\)RIIB/CR2–Ab complex. The amount of Ag acquired thus ceases to depend on the affinity of BCRs for Ag, precluding further affinity maturation.

The weakest link in the chain limits AM

To determine the weakest link, we examined the binding free energies, i.e., \(\Delta G\)'s, of all the links in the chain. The lower (more negative) is the value of \(\Delta G\), the stronger is the link. We assumed that the BCR is tightly anchored into the B cell membrane and is unlikely to be the weakest link.
link. If the BCR were to dislodge from the B cell membrane, no Ag acquisition would occur, and the B cell would not survive. The ΔG’s of anchoring Fc or CR2 receptors in the FDC membrane have not been measured. Theoretical calculations of ‘water-to-membrane’ transfer free energies are reported in the membranome database,[40,41] which provide estimates. Accordingly, the ΔG of FcyRIIB receptor anchoring in the FDC membrane is $-106$ kJ mol$^{-1}$ and that of the CR2 receptor is $-182$ kJ mol$^{-1}$. The affinities of the FcyRIIB receptor for the Fc region of Abs have been measured for all the four IgG sub-classes.[42] The affinities lie in the range $K_d = 0.25-2.5 \times 10^5$ M$^{-1}$, which would correspond to ΔG’s in the range $-26$ kJ mol$^{-1}$ to $-32$ kJ mol$^{-1}$ at 37°C. Similarly, the binding of CR2 to its ligands such as C3d is well understood structurally.[43] The reported affinity is $\sim 2 \times 10^6$ M$^{-1}$, which corresponds to a ΔG of $-37$ kJ mol$^{-1}$. Note that multiple C3d molecules can bind to a single IC and interact with complement receptors, potentially increasing the overall affinity. We looked finally at the ΔG of the Ab–Ag interaction. At the start of the GC reaction, the Ab affinities can be low, $\sim 10^4$ M$^{-1}$, yielding ΔG of $-24$ kJ mol$^{-1}$. As the reaction proceeds, the affinity can rise up to $\sim 10^7$–$10^{12}$ M$^{-1}$, corresponding to ΔG’s of $-42$ kJ mol$^{-1}$ to $-71$ kJ mol$^{-1}$ (see Figure 1).

It followed from the above estimates of ΔG’s that at the start of AM, the weakest link could be the Ab–Ag interaction. Thus, when a B cell with a BCR of a higher affinity for Ag than the Ab in the IC presenting the Ag encounters the FDC, the chain would snap at the Ab–Ag interaction, dissociating the Ag from the IC and resulting in Ag acquisition by the B cell (progressive AM regime in Figure 3B).

As AM progresses, the selection stringency for B cells in the GCs increases steadily due to Ab feedback.[29,30,32,44] Abs produced by recently differentiated plasma cells can traffic back to the GC.[29,30,32,44] They can bind Ag presented on FDCs and mask them from B cells,[29,45,46] rendering Ag acquisition difficult. Further, if their affinity for Ag is higher than the Abs presenting Ag as ICs on FDCs, they can replace the latter Abs and themselves present Ag as ICs (IC turnover) as shown experimentally.[29] Specifically, when IC turnover on FDCs was observed in mice, rapid replacement of endogenous Abs in ICs by exogenous Abs, administered passively, was observed when the latter had higher affinities for the Ag than the former, whereas little replacement was observed when the latter had lower affinities.[29,30,44] Furthermore, a model of the GC reaction incorporating Ab feedback and IC turnover explained several experimental observations of Ab AM.[32] BCRs must form ternary complexes with ICs on FDCs and extract Ag from the ICs,[32] a process that would require the dissociation of the IC.[47] As the affinity of the feedback Abs rises due to AM, Ag extraction by BCRs becomes increasingly difficult.[29,32]

Ab feedback would thus result eventually in the Ab–Ag interaction becoming stronger than the FcyRIIB/CR2–Ab interaction. When this happens, the latter link becomes the weakest, and the chain would snap there (saturating AM regime in Figure 3B).

The FcyRIIB/CR2–Ab interaction is decoupled from the Ag–Ab interaction and is thus not likely to be sensitive to Ab mutations that can increase Ag–Ab affinities (assuming weak allosteric effects). Thus, all BCRs with affinities higher than the FcyRIIB/CR2–Ab interaction would acquire Ag by snapping the FcyRIIB/CR2–Ab complex, leaving little selective advantage for BCRs with higher affinities. The Ag–Ab affinity ceiling would thus be determined by the affinity of the FcyRIIB/CR2–Ab complex.

The weakest link hypothesis thus offers a potential explanation of the ceiling on Ab affinities realized in vivo. We examined next whether the ceiling could be quantified based on the strength of the weakest link and whether it could explain the observations of Abs affinities in vivo.

**THE WEAKEST LINK QUANTIFIES THE AFFINITY CEILING AND EXPLAINS THE AFFINITIES OBSERVED IN VIVO**

We recognized that the ceiling would depend not only on the intrinsic strength of the weakest link identified above but also on the configuration of Ag presentation and acquisition, defined by the tethers holding the Ag and the BCRs pulling it, as well as the force applied by the GC B cell. We considered various scenarios likely to represent the configurations in vivo and quantified the ceiling in each scenario. We note that the Ab–Ag affinities mentioned above correspond to the case where the two proteins involved are in solution and interact in three dimensional (3D) space, which is consistent with the way affinities are typically measured and reported. During Ag acquisition, the proteins are restricted to cell surfaces and thus effectively interact in 2D. Formal ways have been developed to map affinities in 3D to those in 2D and vice versa,[39] These maps rarely change the rank ordering of the affinities,[39,48,49] The weakest link based on 3D affinities would thus remain the weakest link in situ. Our analysis below is thus based on 3D affinities that have the additional advantage of enabling direct comparisons with experiments.

**Bivalent Ag may underlie the low affinity ceilings observed**

We considered first the simplest scenario where AM occurs for an Ag that has two identical Ab binding sites that are accessible simultaneously but are sufficiently separated that each site can be accessed by a single Ab/BCR. We assumed that B cells of a single lineage evolve and result in AM. This scenario, illustrated in Figure 3A, is similar to Figure 3B but with the purple and green epitopes being identical as are the lineages of the corresponding Abs/BCRs. When the ceiling is reached, the presenting Ab in the IC as well as the BCR on the interacting B cell will have affinities for the Ag just above the strength of the weakest link, the FcyRIIB-Ab or the CR2-Ab complex, ‘s’, the latter in the range of $10^4$–$10^6$ M$^{-1}$. The ceiling would thus be $10^4$–$10^6$ M$^{-1}$, corresponding to the snapping of a single FcyRIIB-Ab or CR2-Ab complex (Figure 3B).

We next considered the scenario where the Ag is bivalent, but the epitopes are distinct, i.e., the purple and green epitopes in Figure 4B are not identical. AM would now have to occur simultaneously for the two epitopes, with two distinct lineages of B cells—expressing purple and green BCRs, respectively—evolving for the two epitopes. This is because a B cell with a green BCR lineage, targeting the green epitope,
FIGURE 4  Affinity ceiling under different Ag presentation scenarios. Bivalent Ag with (A) identical, and (B) distinct epitopes, tethered to the FDC by a single FcγRIIB/CR2 receptor. Saturating and progressive AM regimes for (A, B) are depicted in Figure 3B. (C) Multivalent Ag presented by two Abs tethered to distinct FcγRIIB/CR2 receptors, along with progressive (left) and saturating (right) AM regimes illustrated for this configuration. (D, E) Scenarios where the presenting Ab in the IC is linked to the FDC surface by two FcγRIIB/CR2–Ab tethers. (F) A hypothetical scenario with a highly networked topology of FDC receptors, Abs, Ag and BCRs.

The above estimates of the ceiling are based on ΔG’s in the absence of pulling forces. B cells are known to exert a pulling force for Ag acquisition. Mechanical forces can change the on and off rates in complex ways. Descriptions of how each of the links in our chain behaves under tensile forces are not available. Here, we therefore employed phenomenological models, based on...
Kramer’s theory\(^{[58,59]}\) to examine the influence of the pulling force on the affinity ceiling assuming the links to be slip bonds (Box 1). We found that the pulling force typically used by GC B cells did not alter the ceiling substantially.

These bivalent scenarios (Figure 4A,B), and their corresponding lower affinity ceilings, may explain the affinities of Abs smaller than \(10^4\) M\(^{-1}\) observed in vivo (Figure 1B).

**Multivalent Ag encompasses the range of affinity ceilings observed**

We next considered the more complex scenario where Ags contain multiple Ab binding sites (or epitopes) and can bind Abs that tether them to the FDC surface via multiple receptors (Figure 4C–F). The simplest of these scenarios is when an Ag has three identical Ab binding sites, all simultaneously accessible but sufficiently separated that each is accessible to a distinct BCR/Ab and where each Ab is tethered to the FDC via a single Fc\textsubscript{RIIB} or CR2 receptor (Figure 4C). Such multivalent Ag with identical Ab binding sites have recently been designed as potent vaccines against viruses such as HIV\(^{[62–65]}\), SARS-CoV-2\(^{[66,67]}\), and RSV\(^{[68]}\). We again assumed a single B cell lineage evolving, so that the Abs in the presenting ICs and the BCR acquiring Ag have the same affinities when the ceiling is reached. The ceiling would thus be \(10^8\) to \(10^{12}\) M\(^{-1}\). Future studies may refine these estimates using more detailed calculations of the influence of pulling forces\(^{[53,59]}\) or sophisticated steered molecular dynamics simulations\(^{[57,60–62]}\).
is achieved when the affinities of BCRs from each of the B cell lineages exceed $\kappa^2$. The ceiling would remain $10^{8} - 10^{12}$ M$^{-1}$ in all other configurations where two tethers would have to be broken for Ag acquisition (e.g., see two such configurations in Figure 4D and E). This affinity ceiling is consistent with the high affinity ceilings observed in vivo (Figure 1B).

In general, we can imagine Ag being held by \( \Phi \) tethers and tugged by \( \beta \) BCRs (Figure 4F). The ceiling is reached when the collective affinity (or avidity) of the tethers equals the total affinity of the bound BCRs. If the affinity of a BCR is \( \kappa \) (\( = K_A \)), the balance implies $x^\Phi = \rho^\beta$, so that the ceiling becomes $\rho^\beta x^\Phi$, with $x = 10^{6} - 10^{8}$ M$^{-1}$. In Figure 4F, $\Phi = 3$ and $\beta = 2$, thus leading to a ceiling of $10^{6} - 10^{9}$ M$^{-1}$. The ceiling would rise as $\Phi$ increases and $\beta$ decreases, and many combinations of $\Phi$ and $\beta$ could lead to the same affinity ceiling. Our formalism can thus be generalized to multivalent Ag and cross-linked BCR-Ag configurations. We speculate here that scenarios with $\Phi/\beta$ beyond 2 are either sterically hindered, or make the force required for Ag extraction so large that B cells switch to an affinity-independent enzymatic Ag extraction pathway.\(^{[33,69]}\) thus restricting the ceiling to $10^8 - 10^{12}$ M$^{-1}$ (Figure 1B). As the ratio $\Phi/\beta$ decreases and approaches 1, the ceiling also decreases and approaches $\kappa$. In some experiments with large Ag, a ceiling of $\sim 10^5$ M$^{-1}$ has been observed.\(^{[70]}\) and may correspond to configurations where $\Phi = \beta$, so that the affinity ceiling is approximately $\kappa$. Mechanical pulling forces did not alter these estimates significantly (Box 1).

Our estimates of the ceiling thus explain the range of Ab affinities realized in vivo.

**STRENGTHS AND IMPLICATIONS OF THE WEAKEST LINK HYPOTHESIS**

**Experimental evidence exists that supports the weakest link hypothesis**

The ability to explain the observed Ab affinities in vivo provides strong support to our hypothesis. Specifically, the range of values of the ceiling we estimated, $\sim 10^6 - 10^{12}$ M$^{-1}$, arising from the various Ag acquisition configurations we envisioned, is consistent with the range of affinities observed in vivo (Figure 1B). Further, evidence of the snapping of such Ag acquisition chains at their weakest links comes from in vitro experiments. In elegant experiments, Spillane and Tolar\(^{[33]}\) attached Ag covalently via DNA and streptavidin tethers to two kinds of surfaces: (1) stiff and immobile and (2) flexible and partly mobile. B cells interacted with this Ag, thus, through a chain of three complexes: Ag-DNA, DNA-streptavidin and streptavidin-surface. Ag extraction by B cells was compared between the stiff and the flexible surfaces. When Ag was presented on the stiff surface, B cells preferentially ruptured the weaker DNA-streptavidin complex and acquired Ag. However, with the flexible surface, which appeared to be the weakest link, the entire chain of complexes along with an annular surface patch was internalized. Further, the latter scenario resulted in similar amounts of extracted Ag bound to either weak or strong DNA tethers, indicative of a loss of affinity discrimination. These observations support the weakest link hypothesis and the consequent emergence of an affinity ceiling.

**Alternative hypotheses cannot satisfactorily explain the ceiling**

We examined several alternative hypotheses that could potentially explain the origins of the ceiling and found them wanting. One hypothesis is that AM ends prematurely because infections can get cleared rapidly and offer inadequate time for B cells to evolve. Note that the ceiling refers to the highest affinities seen in vivo. In many situations, the maximum affinities realized are far below the ceiling (Figure 1B). Shortage of time, however, appears not to be a satisfactory explanation because the ceiling is seen also with chronic infections such as HIV-1, which can last tens of years. The evolution of HIV-1 bNAbs is known to take years\(^{[45]}\) and yet the affinities of bNAbs do not breach the ceiling (see Figure 1A). Recent studies have argued that chronic infections can result in more potent GC responses.\(^{[71,72]}\) Yet, it is possible in some chronic infection settings that a larger fraction of selected GC B cells than in acute infections is terminally differentiated into plasma and memory cells, which may leave insufficient numbers of GC B cells to efficiently mutate and continue the GC reaction.\(^{[73]}\) possibly contributing to the low ceiling. This hypothesis, however, cannot explain the scenarios where Abs with high affinities, close to the ceiling, arise soon after the onset of (an acute) infection due to random mutations in the Ab genes.\(^{[24]}\) Yet, as the infection progresses, the affinities saturate and do not rise above the ceiling.\(^{[24]}\) Indeed, it is this observation that prompted Foote and Eisen to posit the existence of an affinity ceiling.\(^{[11]}\)

Another hypothesis is that the ceiling arises from continuous Ag evolution. A persistent evolutionary arms race between pathogens or tumour cells and the host immune system is characteristic of infections with rapidly mutating pathogens such as HIV and hepatitis C virus\(^{[74–79]}\) and cancer.\(^{[80]}\) HIV-1 genomic diversification within infected individuals continues over many years.\(^{[75,81–83]}\) Failure of drugs and vaccines has been attributed to such mutational escape, often aided by recombination.\(^{[76,79,84–91]}\) It is conceivable in such a scenario that Ab evolution, due to B cell selection, increases Ab affinity for a target Ag, whereas Ag evolution compromises the affinity by altering the target. A balance between these competing effects may define the ceiling. This explanation, however, fails to describe how the ceiling arises with simple, non-mutating Ag such as hapteners, nor does it explain why in vitro selection can allow Ab affinity to breach this ceiling.\(^{[9]}\) The high in vitro affinities also rule out intrinsic limitations in Ab structures precluding the realisation of higher affinities for Ag than the ceiling.

Yet another hypothesis is that the ceiling arises from the limitations of Tfh cell help in GCs. Multiple GC B cells are thought to interact simultaneously with an individual Tfh cell, causing the Tfh cell to polarize preferentially in the direction of the B cell presenting the most Ag.\(^{[2,92]}\) The latter B cell, in turn, receives survival signals from the Tfh cell and is selected at the expense of the other interacting B cells. One can imagine that the contact area between individual Tfh cell-B cell pairs is...
limited and can accommodate a maximum of $\theta$ Ags. If, due to receptor aggregation, two B cells that have acquired different amounts of Ag from FDCs can accumulate $\theta$ Ags in their $T_h$ cell contact areas, the $T_h$ cell would not be able to discriminate between the two lateral diffusion of receptors could trigger such accumulation.[148,93] This could also be a mechanism giving rise to an affinity ceiling. A limitation of this argument, however, is its inability to explain why the ceiling is always lower than $10^{10} - 10^{12}$ M$^{-1}$ in vivo. We note that any other hypothesis, too, which can conceivably give rise to a ceiling must be able to explain the latter limit. In our hypothesis, the ceiling is linked to the strengths of the tethers holding Ags on FDC surfaces, which explains the latter limit.

Thus, our explanation, together with the supporting experimental evidence above, appears to be the most plausible.

**Strategies to test the weakest link hypothesis and improve vaccination**

Ab-mediated immune responses depend strongly on the affinity of the Abs for their target Ag.[114–17] In many commonly encountered infections that we typically naturally fight off, our endogenous Ab responses appear potent despite the affinity ceiling. It is possible, thus, that the ceiling may have evolutionary underpinnings in the Ab affinities necessary to survive common infections (Box 2). In such situations, particularly when Ab concentrations are large enough to bind and neutralize most Ag, an increase in the ceiling may be unwarranted. Yet, in many modern infections, such as with HIV-1, the ceiling may be unduly restrictive, especially when neutralising Ab concentrations may be suboptimal. Only ~1% of HIV-1 infected individuals produce potent bNAb, after years of infection.[94,95] Most bNAb target conserved regions of HIV-1. Several reasons have been suggested for the poor and delayed elicitation of bNAb, including specific B cell or T cell precursor frequency,[92,96] virus evolution,[4,5] high degree of required somatic hypermutation,[95,97] and competition between autologous B cells.[98] The ceiling may further contribute to limiting a potent bNAb response. In such situations, vaccination or other interventions that can overcome the limit due to the ceiling may have advantages. Our study suggests possible ways of achieving this. Broadly, because the ceiling arises from the weakest link in the chain of protein complexes mentioned above (Figure 3), strengthening the weakest link would raise the ceiling and hence elicit higher affinity Abs.

One strategy to strengthen the weakest link could be passive immunisation with engineered Abs. Passive immunisation with HIV-1 bNAb improved the endogenous humoral response to HIV-1.[101] A plausible mechanism underlying this improvement is that the passively administered Abs enter GCs via the Ab feedback mechanism and improve selection stringency.[29,32] Modifying the Fc regions of the passive Abs to increase their FcRIIB binding affinity and/or using tight binding IgG subtype[42] would strengthen the weakest link and enhance AM, offering a direct test of our hypothesis. This strategy is
similar to introducing mutations in the Fc regions that would increase Ab affinities for the FcRn receptor, and therefore their circulation half-life; such engineered Abs have been found safe.[102,103]

Mutagenesis of FcRIIB shows that such modifications are possible.[104] An Ab with mutations in the Fc region showed an ∼200-fold higher affinity for the FcRIIB receptor.[105] Several studies have explored CR2 and C3d mutants that alter the CR2-C3d interaction.[43,106] Corresponding changes in the ceiling would further validate our explanation. It would also be interesting to examine whether the humoral responses of individuals with clinically relevant, naturally occurring polymorphisms of the FcRIIB receptor exhibit different affinity ceilings.

CONCLUSIONS AND OUTLOOK

The origin of the ceiling on Ab affinities in vivo has been unresolved for decades,[11,12] challenging our understanding of AM and potentially compromising vaccine design. Here, we argued that the weakest link in the chain of protein complexes involved in Ag acquisition by B cells in GCs defines this limit. Using available estimates of the affinities of the complexes and considering various configurations of Ag presentation and acquisition, we showed that the resulting affinity ceiling could explain the range of Ab affinities realized in vivo. A new conceptual understanding of AM thus emerges.

Our explanation of the affinity ceiling is similar in spirit to that offered first by Foote and Eisen.[11] The ingenuity of their explanation was in its recognition that the ceiling would arise from processes extrinsic to specific Ag-Ab interactions. With soluble Ag, these processes were the rates of diffusion of Ags and the rate of internalisation of Ag bound to BCRs on specific B cells. With surface tethered Ag that we considered, the ceiling comes from the FcRIIB-Ab or CR2-Ab interactions. In vivo, because Ag acquisition in GCs is predominantly from the FDC surface, our explanation is expected to prevail.

Evidence in support of the hypothesis exists in in vitro experiments. The hypothesis could be tested in vivo by using passive immunisation with Abs containing mutations in the Fc regions that increase their FcRIIB binding affinity, strengthening the weakest link. Variations in the humoral responses across individuals carrying natural variations in these regions could provide another test. Strengthening the weakest link, including by mutating the Fc regions of passively administered Abs, may lead to improved vaccination and immunotherapy designs, with possible implications for our ability to combat infectious diseases and cancers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rajat Desikan conceptualized the study. Rajat Desikan, Rustom Antia and Narendra M. Dixit analysed results and co-wrote the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Rajat Desikan https://orcid.org/0000-0002-0785-8187
Narendra M. Dixit https://orcid.org/0000-0002-2145-9828

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