Breaking the affinity ceiling for antibodies and T cell receptors

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The antigen receptors made by lymphocytes are antibodies, which exist as soluble and cell-bound molecules, and T cell receptors (TCRs), which are always found on cell surfaces. The function of these receptors in immunity depends on their specificity and affinity for antigen. Specificity—the potential to bind one unique chemical structure more strongly than a number of similar alternatives—is established by antibody and TCR gene rearrangements early in a lymphocyte's ontogeny. Affinity—the equilibrium constant for antigen complexation (which we express as a dissociation constant, K_d)—can increase radically in the antibody-producing B lymphocytes as a result of somatic hypermutation of antibody genes and selection of cells with improved binding phenotype (1, 2), an antigen-driven process known as affinity maturation. Although the extent of somatic hypermutation of rearranged TCR genes remains controversial (3), affinity maturation of T lymphocytes at the level of cell populations by selection of the available repertoire recently has been described (4).

The affinities of antibodies and TCR obtained *in vivo* tend to fall within characteristic ranges constrained by biological requirements imposed during the ontogeny of B and T cells, about which more will be said later. Affinity maturation *in vitro*, as a rule, is not subject to the same biological constraints. In particular, affinity ceilings observed *in vivo* should not apply. The affinities of exhaustively *in vitro* matured antibodies or TCR should instead approach either a methodological ceiling intrinsic to the specific maturation protocol used or a molecular ceiling intrinsic to the architecture of antibodies and TCR. Two papers in PNAS describe *in vitro* affinity maturation of a TCR and an antibody using an ingenious system of yeast surface display (5, 6). *In vitro* TCR affinity maturation has not been reported previously. *In vitro* affinity maturation of antibodies has been described by several groups (7, 8), but never with so spectacular an endpoint, a K_d of 5×10^{-14} M. In both the TCR and antibody cases the affinities ultimately obtained are orders of magnitude beyond the affinity ceilings observed *in vivo*. In this commentary we compare the *in vivo* affinity constraints with the *in vitro* constraints of the yeast system and comment on how breaking the affinity ceiling may affect basic immunology and immunotherapy.

Yeast Display

Boder and Wittrup (9) several years ago developed a yeast-based system for surface display of recombinant proteins. The main element of this system is a fusion of the recombinant gene to the AGA2 gene of *Saccharomyces cerevisiae*. The AGA2 fusion protein is secreted and attaches through two disulfide bonds to the AGA1 gene product, which is covalently linked to the fibrillar layer of the yeast cell wall (10). This arrangement leaves the recombinant fusion protein on the outside of the cell, hence biochemically selectable, and the gene encoding the fusion protein on a plasmid inside the same cell, hence recoverable. The yeast can display thousands of copies of the recombinant fusion protein per cell and can be stained with fluorescent ligands and sorted by flow cytometry.

Antibodies

Besides recognizing antigen through their surface antibodies, B cells can internalize and fragment protein antigens and present antigen to T cells in the form of peptides bound in the groove of MHC molecules. Specific antigen recognition by a surface antibody on a B cell, followed by endocytosis, leads to efficient antigen presentation (11), initiating cytokine release, B and T cell proliferation and differentiation, somatic hypermutation, and so on. We argued in an earlier commentary (12) that the residence time of an antigen complexed to a B cell surface antibody would constrain the dissociation rate constant (k_{off}) selectable *in vivo*. The kernel of our proposal was that antibody-antigen complexes with lifetimes much longer than the time necessary for uptake would all be processed equally well, hence could not be distinguished. Batista and Neuberger (13) recently demonstrated that this is so. They cocultured antilysozyme antibody transfectomas with lysozyme-specific T cell hybridomas, added soluble lysozyme at various concentrations, and monitored IL-2 release by the hybridomas as an indicator of antigen presentation. The lifetime of the antibody-lysozyme complex was varied by mutations in either the lysozyme or antibody. The result was that antibody-lysozyme pairs with a bound lifetime of 12 min or less showed an IL-2-vs.-antigen response that was very sensitive to k_{off}, whereas all pairs with a longer lifetime behaved identically regardless of k_{off}.

Residence time of an antigen bound to a surface antibody is also a key parameter in the *in vitro* affinity maturation system, although the antigen is not internalized by the yeast cells. In this method, yeast expressing a library of mutant antibodies are first saturated with fluorescent antigen, then treated with a nonfluorescent competitor antigen. Excess competitor makes dissociation of the fluorescent antigen effectively irreversible, and gradually the labeled cells lose their fluorescence. The time the competition is allowed to continue is critical. If too short, fluorescence differences between the wild type and a slower-dissociating mutant will be indistinct. If too long, all cells will be equally nonfluorescent. Boder and Wittrup (14) developed an elegant mathematical foundation for an optimal selection strategy. Given some typical experimental conditions and a desire for moderate stepwise improvements in k_{off}, the optimal incubation time for a selection step is approximately $5/k_{off}$. Boder *et al.* (6) used this model assiduously in the antibody *in vitro* affinity maturation work. They made kinetic measurements at each of four rounds of mutagenesis and selection of an anti-fluorescein antibody and adjusted compe-

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tition times and flow sorting parameters accordingly. Their results beautifully match the theory, and antibody-hapten stabilities of selected mutants march in clusters down a k_{off} plot, with regular kinetic improvements at each round of mutagenesis and selection. In the final round, incubation of labeled cells with nonfluorescent competitor continued for 5 days, a far longer interval than the 12-min window observed *in vivo* (13). Remarkably, the improvements in k_{off} and in affinity showed no sign of reaching a plateau in the last round. Therefore, neither an intrinsic molecular ceiling for antibody-hapten affinity nor a methodological ceiling inherent in the mutagenesis and selection protocol was reached. However, the methodological ceiling must be near: a further round of affinity maturation would require incubation with competitor to last on the order of a month. During this time the yeast would have to remain viable without dividing, and the surface antibody would have to resist unfolding.

TCRs

The TCRs we discuss here are the $\alpha\beta$ heterodimers present on most T cells. The natural ligands for these receptors are peptide-MHC (pepMHC) complexes on the surface of other cells with which they are in contact. The peptides normally are derived by intracellular fragmentation of proteins, including indigenous or pathogen-encoded protein, but the MHC molecules are encoded by the host genome. Antigen recognition by T cells can result in various protective functions, including (*i*) destruction of virus-infected cells by cytolytic T lymphocytes that express the CD8 coreceptor, and (*ii*) release by various T cells expressing the CD4 coreceptor, of cytokines that either enhance antibody production by B cells or cause local inflammation.

Before discussing limits on TCR affinity *in vivo*, we note that evaluation of TCR-pepMHC affinity and its significance is beset with methodological and conceptual problems. For one, the value of a TCR-pepMHC affinity constant depends on the way the measurement is made. TCR affinity values generally are determined with monomeric soluble pepMHC complexes either with the TCR having also been obtained in soluble form and immobilized on a biosensor chip ("cell-free" affinity), or with the TCR in its natural state as an integral membrane protein on live T cells. With live T cells, K_d values (intrinsic affinity) have been found to vary from slightly above 100 μM to 0.1 μM. In cell-free systems the values tend to be weaker. One difference is the absence in the cell-free system of the CD8 coreceptor found on cytotoxic T lymphocytes. CD8 interacts with a conserved MHC domain on target cells at a K_d of 10^{-4} M, and the free energy of this interaction could boost the observed affinity of TCR for pepMHC (15, 16). Second, specific T cell responses to the pepMHC they recognize (also termed epitopes) on target cells appear to be determined by affinity of the TCR for its epitope and the number of copies of the epitope per target cell (epitope density). The epitope density is a fundamental parameter for interpreting TCR affinity as it applies to T cell function, yet epitope densities are difficult to measure and only a few values have been directly determined. The least ambiguous approximations come from cell lines expressing surface MHC molecules having empty peptide-binding sites. Extracellular peptides can bind to these MHC molecules, thereby creating epitopes on target cells. For peptides that react similarly with any particular MHC, relative epitope density values thus have been estimated from the concentration of free peptide needed to elicit a T cell response of a particular magnitude (e.g., half-maximal lysis of target cells in cytolytic reactions).

A TCR epitope includes both the antigenic peptide and adjacent regions of the MHC molecule's peptide-binding site. In developing the capacity for this dual recognition, T cell precursors in the thymus (thymocytes) undergo two processes that bear on the affinity ceiling for antigen recognition. T cell precursors able to recognize thymic (self) peptides bound to MHC are positively selected, in that they continue to develop. Perhaps 95% of cells fail this test and are eliminated, but the remainder are imprinted with an ability to recognize one or another of the host's own MHC molecules (17, 18). Negative selection in the thymus eliminates positively selected cells that react too strongly with thymic pepMHC and thus could become dangerously autoreactive. The positive-negative selection model defines a window in which thymocytes die if TCR engagement by self-pepMHC exceeds some upper affinity limit and fail to survive if below some lower limit (19). The actual limits have not been quantified. The affinity cutoff for negative selection in the thymus is the first of three *in vivo* TCR affinity ceilings we discuss.

The second affinity ceiling applies to mature T cells in encounters with antigen and is the intrinsic affinity sufficient for recognition of a vanishingly low epitope density, approaching 1 epitope per target cell. When TCR affinity is at the upper limit of the values determined on live cells (K_d 0.1 μM), low epitope densities can elicit a strong T cell response with free peptide concentrations between 1 and 0.1 pM and fewer than 10 epitopes per target cell. Many orders of magnitude higher free peptide concentrations are required to elicit similar responses when the TCR affinity is low, e.g., 100 μM. As with B cells, T cells must engage antigen to survive, proliferate, and differentiate into memory cells. T cells able to respond to low epitope density will have a survival advantage over T cells that remain unreactive until high epitope density is reached. The reciprocal relation between epitope density and TCR affinity has an endpoint at <10 pepMHC molecules per target cell and TCR intrinsic K_d 10^{-7} M (20, 21). At this endpoint so few epitopes per target cell are required that the efficacy of T cells in cytolytic reactions would not be enhanced by higher intrinsic affinity values (20).

A third *in vivo* TCR affinity ceiling is possible (22). A passive limit for TCR affinity might be envisioned, in which, say, 10^{-7} M is the maximum selectable affinity. Higher affinity clones arise, but with no reason for these clones to dominate the repertoire, are not detected. Alternatively, very high affinity—the result, say, of a very slow k_{off}—might be a disadvantage, and such T cells may fail to proliferate or even be actively eliminated, entailing a third affinity ceiling. Valitutti *et al.* (22) found that one pepMHC on a B cell can engage and downregulate many TCR on a T cell hybridoma. The serial engagement of TCR molecules on a T cell by pepMHC on a target cell seems to be necessary for specific T cell responses, suggesting that transient signaling by many receptors gives a greater stimulus to cellular activation than constant signaling by a single receptor. If so, a clear rationale for an off-rate limit would be that an inability for pepMHC to disengage from one TCR and rebind another would render a T cell less able to activate than one with a better balance between k_{off} and k_{on}.

In another type of recognition model, kinetic proofreading (23, 24), TCR-pepMHC engagement is coupled to an exergonic specificity-validating process. This means of increasing biological specificity originally was formulated around the fidelity of protein synthesis, in which amino acid side chains of similar chemical structure must be distinguished (25). Such a model is attractive for T cell antigen recognition because the interaction of TCR with self-MHC and accessory molecules such as CD8 contribute to affinity without contributing to specificity, and the actual free energy of binding caused purely by the antigen is low. Kinetic proofreading is much more efficient if recognition of a cognate structure is not only enhanced, but inappropriate recognition of a noncognate structure is penalized (26). Models reformulated for T cell antigen recognition that embody this princi-

ple (25) can account for many observed phenomena, including antagonism of recognition by altered peptides. Such models typically equate long TCR-pepMHC complex lifetime with valid recognition and short lifetime with invalid recognition, respectively, yielding positive and negative signals for T cell activation. The import for a third affinity ceiling is that the optimal k_{off} is slow enough to guarantee sufficient time for the specificity validation process to initiate a positive signaling cascade. Still slower values are not an advantage, but neither are they a disadvantage unless a serial engagement mechanism operates as well.

Some recent studies contend that it is not the affinity of TCR-pepMHC interactions but the dissociation rate of the TCR-pepMHC complexes formed by this interaction that determine the T cell's response. The signal transduction reactions triggered by TCR ligation are unlikely to approach equilibrium, and the outcome of any particular reaction is indeed likely to depend on the stability of intermediate complexes. But TCR ligation by pepMHC, the initial step in T cell activation, has been shown to approach equilibrium (27), and thus the average number of TCR-pepMHC complexes formed in a T cell-target cell encounter is determined by the equilibrium constant. It is this number, together with the first-order dissociation of these complexes, that determines how many complexes survive long enough to trigger the next step in T cell activation. Thus it is not a question whether T cell responses are driven by k_{off} or affinity: both are linked in the initial TCR ligation reaction upon which specific T cell responses depend.

Holler *et al.* (5) derived a high-affinity TCR using yeast surface display methods analogous to those described above for *in vitro* antibody maturation. Starting with a temperature-stabilized single-chain TCR from the receptor of an extensively studied T cell clone called 2C, they selected from a library of 10^5 yeast clones 15 mutants having higher affinity for a particular pepMHC complex. The affinity of the most reactive mutant (9 nM) was about 100 times greater than that of the original receptor.

Concluding Remarks

The overall lesson from antibody and TCR *in vitro* affinity maturation is that mechanisms generating these molecules *in vivo* operate in an affinity regime far below the inherent potential of antibodies and TCR for ligand binding. The most significant aspect of *in vitro* affinity maturation of antibodies is perhaps practical, in that escape from the *in vivo* affinity ceiling may make possible an improved set of reagents for diagnosis and therapy. Breaking the TCR affinity ceiling has great practical ramifications as well, but equally significant are the possibilities created for testing fundamental hypotheses of T cell antigen recognition. The potential usefulness of high-affinity TCR is evident from the ability of the affinity matured 2C to specifically stain cells that display the appropriate pepMHC complexes. High-affinity TCR thus might provide specific reagents for measuring epitope densities. If they can be expressed on T cells, they also make it possible to determine an affinity limit above which T cell responses are impaired. They also could serve as useful reagents to detect particular pepMHC complexes important for stimulating the autoreactive T cells involved in autoimmune disorders.

A soluble molecule consisting of a TCR-like recognition component and a toxic effector component could be useful therapeutically for certain autoimmune or infectious or neoplastic diseases. The weak affinity of the currently available TCR has been a barrier to development of such fusion proteins for therapy. Their 0.1 μM ceiling is in the range of only the weakest antibody affinity acceptable for therapy (28). The work by Holler *et al.* (5) clearly removes this barrier. However, another barrier arises from MHC polymorphism. A TCR specific for a particular epitope in one patient may be ineffective in others lacking that MHC, either because their MHC molecules do not bind the peptide or the resulting pepMHC complexes are not recognized by that TCR. And a third barrier may exist if a soluble high-affinity TCR were to react strongly with foreign (allogeneic) MHC—the phenomenon called alloreactivity. Nevertheless, under circumstances where a particular MHC molecule occurs frequently and its associated pathogenic peptide is known (e.g., HLA-DR4 and an encephalitogenic peptide in multiple sclerosis), a high-affinity TCR derived from a representative patient's T cells conceivably could prove useful in ablating undesirable antigen-presenting cells in many other affected individuals. Overall, high-affinity TCR generated by yeast display could provide the starting point for the development of novel diagnostic and therapeutic agents.

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