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Design and Engineering of Deimmunized Biotherapeutics

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Abstract

Therapeutic proteins are powerful next-generation drugs able to effectively treat diverse and devastating diseases, but the development and use of biotherapeutics entails unique challenges and risks. In particular, protein drugs are subject to immune surveillance in the human body, and ensuing antidrug immune responses can cause a wide range of problems including altered pharmacokinetics, loss of efficacy, and even life-threating complications. Here we review recent progress in technologies for engineering deimmunized biotherapeutics, placing particular emphasis on deletion of immunogenic antibody and T cell epitopes via experimentally or computationally guided mutagenesis.

Graphical Abstract

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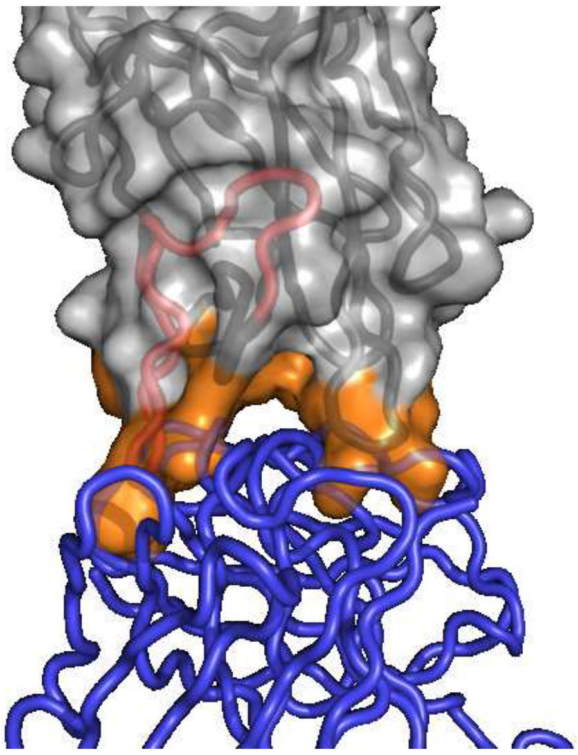
Conflict of interest statement

Karl E. Griswold and Chris Bailey-Kellogg are Dartmouth faculty and co-members of Stealth Biologics, LLC, a Delaware biotechnology company. These authors acknowledge a potential conflict of interest related to their associations with this company, and they affirm that their above cited works are free of any bias. This article has been reviewed and approved as specified in their Dartmouth conflict of interest management plans.



Introduction

Therapeutic proteins represent the cutting edge of modern medicine, and advances in biotechnology are driving growth in the biopharmaceuticals market.[1] As biological entities, however, proteins are subject to immune surveillance in the human body,[2,3] and the induction of antidrug antibody responses can result in a wide range of sequelae including altered pharmacokinetics, loss of efficacy, and more dangerous complications such as hypersensitivity and anaphylactic responses, cross-neutralization of endogenous proteins, and deposition of toxic immune complexes.[4,5] Given the detrimental consequences of anti-biotherapeutic immune responses, there is growing consensus among regulators, physicians, and the biopharma industry that fully exploiting these powerful drugs requires assessment and mitigation of immunogenicity risk.[6-10]

A protein's immunogenic potential is a complex function of diverse interacting factors.[4,10,11] Thus a variety of different approaches have been pursued to mitigate immunogenicity, including shielding proteins with chemical or biological blocking moieties (e.g., PEGylation,[12,13] XTENylation,[14] PASylation,[15] or reductive methylation[16]), explicitly training the immune system to tolerate proteins,[17] or implicitly rendering proteins tolerable by humanization (with emerging new engineering techniques for antibodies[18-22] as well as non-immunoglobulin proteins[23,24]). In any case, molecular recognition of exogenous proteins by antibodies, antigen presenting cells, and T cells is central to the anti-biotherapeutic immune response, and this review focuses on protein deimmunization by genetic manipulation of immunogenic subsequences, termed "epitopes".

Strategies to deimmunize a protein by mutagenically “deleting” its epitopes are grounded in a detailed understanding of the cellular and molecular mechanisms underlying the antidrug immune response. There exist two very distinct types of epitope. Antibody epitopes are comprised of solvent exposed amino acids on the intact protein, and they constitute the binding sites for B cell receptors and soluble antidrug antibodies (Fig. 1A). T cell epitopes, in contrast, are short peptide fragments proteolytically processed from biotherapeutics (Fig. 1A & B), and they play a key upstream role in the antidrug antibody response. Briefly, a patient’s professional antigen presenting cells can internalize putative protein antigens and degrade them into peptide fragments. Immunogenic peptides, termed T cell epitopes, are then surface displayed via class II major histocompatibility complex molecules (MHC II or HLA in humans), where they can interface with surface receptors on cognate $CD4^+$ helper T cells (Fig. 1B).[25] This ternary molecular recognition event initiates a signaling cascade that activates T cells, drives B cell maturation, and ultimately leads to production of high affinity antidrug antibodies.[4,26] (It bears noting that antidrug antibodies can be generated independent of T cell help, but these T independent responses are generally lower affinity and perhaps less significant than T cell dependent responses.[4,26])

Antibody Epitope Deletion

Antidrug antibodies recognize and bind to sites on the surface of intact biotherapeutic agents, and these epitopes may be composed of either contiguous amino acid sequences (linear epitopes) or discontiguous residues that are close in space (conformational epitopes). Regardless, the protein-protein interaction surface exhibits shape complementarity and finely tuned energetics. Therefore, appropriate amino acid substitutions in a biotherapeutic can disrupt these binding interfaces and effectively delete antibody epitopes.[27•] The target epitopes for deletion are established by a range of strategies including simply focusing on hydrophilic surface residues,[28] random mutagenesis and high throughput screening,[29] more precise mapping using panels of antidrug antibodies,[27•,30•] or in rarer cases structure-guided design via antibody-antigen co-crystals.[31] In virtually all cases, however, substitutions that disrupt antibody binding while retaining biotherapeutic function are selected based on scanning alanine or trial and error mutagenesis.

Due to their importance in anti-cancer therapies, which can be undermined by immunogenicity, toxins have been the focus of substantial reengineering efforts to eliminate antibody epitopes. Diphtheria toxin has been deimmunized by mutagenic substitution of lysine, arginine, glutamine, and glutamic acid residues, amino acids which had been shown previously to be critical contributors to antibody epitopes of *Pseudomonas* exotoxin A (PE).[32] Inspection of the diphtheria toxin structure identified 24 target residues on the protein surface, and sequential rounds of mutagenesis and testing produced the 7-mutation variant dDTEGF13, which was found to elicit greatly reduced antidrug antibody titers in both BALB/c and C57Bl/6 mice while retaining potent cytotoxic activity both *in vitro* and *in vivo*.[28] It was presumed that antibody epitopes were deleted, though T cell epitope deletion (see below) was not definitively ruled out. The PE toxin itself has been the subject of extensive and systematic deimmunization efforts. Murine antibody epitopes of PE were mapped and deleted, yielding immunotoxin HA22-LR-8M that had better than wild-type activity *in vitro*, near wild-type efficacy *in vivo*, yet exhibited reduced antibody binding *in*

vitro and reduced immunogenicity in BALB/c mice.[33] These mutations have been leveraged by others to create deimmunized bispecific immunotoxins based on PE.[34] It was subsequently found that human antibodies could bind PE epitopes distinct from those of murine antibodies, prompting a new effort that specifically targeted human antibody epitopes. The resulting variant HA22-LR-LO10 likewise exhibited near wild-type activity *in vitro* and wild-type efficacy *in vivo*, yet demonstrated dramatically reduced binding with human anti-serum from patients previously treated with various versions of the wild-type toxin sequence.[30•] These epitope-deleting mutations have been engineered into immunotoxins that exhibit potent anti-cancer activities,[35,36] and in particular the R06927005 immunotoxin variant (formerly RG7787) has entered human trials.

Recent literature describes several therapeutic enzymes and enzyme cofactors engineered for reduced antibody binding. *E. coli* type II asparaginase, an important therapeutic for acute lymphoblastic leukemia, is one such example. Focusing on a previously identified antibody epitope, the researchers introduced two mutations, K228S and Y176F, that increased cytotoxicity towards leukemic cells, decreased undesirable glutaminase activity, and reduced reactivity with immune serum from both mice and a leukemia patient treated with the wild-type enzyme.[37] Another recent example is deimmunization of factor VIII (FVIII), a life-saving therapy for hemophilia A patients. Guided by a FVIII-antibody co-crystal structure, 43 distinct point mutants within the antibody binding interface were generated, and their binding affinities for known inhibitory monoclonal antibodies (mAb) were measured. Ultimately the F2196K variant, which retained more than 75% wild type activity, was found to evade binding and inhibition by both human and murine mAbs directed against the C2 domain, but it failed to evade a murine mAb directed against the distal A2 domain.[31] This result highlights the challenge inherent to antibody epitope deletion in the context of polyclonal antidrug antibody responses; in the clinic, success will likely require redesign of multiple antigenic surface sites, as described above for the PE toxin. Thus, the F2196K variant represents a proof-of-concept for structure-guided antibody epitope deletion, but additional molecular engineering will be necessary in order to evade the diverse array of inhibitory antibodies that FVIII may elicit.[38]

Other strategies for antibody epitope deletion have leveraged large combinatorial libraries and high throughput screening. For instance, the anti-inflammatory agent chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) has been engineered by directed evolution so as to minimize binding of human antibodies. Randomly mutated and shuffled CHIPS libraries were displayed on phage, panned for binding to their C5a receptor target, and negatively selected for binding with polyclonal human anti-CHIPS antibodies. Following additional rational design, seven variants bearing five to eight mutations each were characterized and found to maintain reasonable biological activity (6- to 10-fold reduction relative to wild-type), good thermostability (typically better than wild-type), and yet exhibited 40- to 190-fold reductions in binding titer with human anti-CHIPS antibodies. [29]

Clinical complications of antidrug immune responses are the result of antidrug antibodies, and intuition therefore suggests that mutagenic deletion of antibody epitopes is a useful strategy by which to design better biotherapeutics. Importantly, the human immune system

(and those of other mammals) is able to generate antibodies against multiple surface epitopes of a given therapeutic protein. See for example studies on the PE toxin, FVIII, and asparaginase [30,32,33,38,39] to name only a few. However, there tends to be substantial binding site overlap among the diverse antibodies that comprise a polyclonal response, and thus a protein's antibody epitopes can be "grouped" into discrete subsets, each of which might be deleted with one or a few mutations.[27•] Moreover, murine model studies with antibody epitope depleted diphtheria and PE toxins show that the mouse immune system does not readily generate new antibodies against neoepitopes during repeated administration of engineered toxin variants.[28,33] These results, and results from earlier studies,[40-43] suggest that antibody epitope deletion may indeed have clinical utility. At the same time, it should be acknowledged that the human immune system is arguably the world's most efficient antibody discovery and affinity maturation engine. As a result, there remains the possibility that repeated administration of antibody epitope depleted variants to human subjects might elicit new antibodies directed against alternative epitopes. To mitigate this risk, another compelling deimmunization strategy targets upstream molecular recognition events within the overall antidrug immune response: deletion of T cell epitopes.

Experimentally-driven T Cell Epitope Deletion

Biotherapeutic deimmunization by T cell epitope deletion has a long and well validated history. Indeed, though not explicitly articulated at the time, removal of immunogenic T cell epitopes was an implicit result of early antibody humanization strategies.[44,45] Deimmunization of staphylokinase, to treat myocardial infarctions, is an early seminal study of explicit T cell epitope deletion,[46] and other early examples include deimmunization of Factor VIII domain C1,[47] erythropoietin,[48] interferon beta,[49] and a beta lactamase enzyme.[50] More recently, the general approach has proliferated and become more sophisticated. One case in point is the systematic application of T cell epitope deletion to various FDA approved monoclonal antibodies that suffer from undesirable immunogenicity.[51•] An important conclusion from this work was the fact that even fully human antibodies, such as the #1 selling drug Humira, can be highly immunogenic due to T cell epitopes within hypervariable CDRs, yet epitopes within such functionally critical regions may be amenable to mutagenic deletion.

A prominent example of increasingly sophisticated T cell epitope deletion strategies can be found in work on *E. coli* type II asparaginase. Guided by bioinformatics prediction of likely T cell epitopes, anchor residues in three putative immunogenic hotspots were subjected to iterative site-directed saturation mutagenesis followed by high-throughput functional screening using a customized flow cytometric assay. Variant enzymes exhibiting high catalytic activity were isolated, and those functional mutations predicted to be disruptive of class II MHC binding were chosen as templates for subsequent rounds of mutagenesis and screening. This neutral drift strategy ultimately produced the 8-mutation variant 3.1.E2 that exhibited high catalytic proficiency (k_{cat} equal to wild-type and only 3-fold higher K_M) but was significantly less immunogenic in HLA transgenic mice expressing the human DRB1*0401 MHC II allele (assessed by both *ex vivo* cellular immunoassays and *in vivo* anti-drug antibody titers).[52••]

Anti-cancer immunotoxins have proven to be another productive space for T cell epitope deletion. For example, extensive experimental efforts have generated a detailed T cell epitope map for the PE toxin. Using immune cells from both healthy donors and patients previously treated with the immunogenic PE38 variant, eight immunogenic regions were identified and subsequently deleted via a combination of domain truncation and mutagenic substitution of critical epitope anchoring residues. The resulting deimmunized variant LMB-T18 exhibited high *in vitro* cytotoxic activity, potent *in vivo* anti-tumor activity, and yet it decreased *ex vivo* human T-cell activation by 90% compared to the native immunotoxin.[53••] Bouganin is another highly potent toxin with potential utility in cancer therapy, though as a plant protein it runs the risk of eliciting detrimental immune responses. Experimental epitope mapping and trial and error mutagenic T cell epitope deletion yielded the engineered variant de-bouganin, which has demonstrated low immunogenicity and high anti-tumor activity in a large number of preclinical and clinical studies. In human subjects the VB6-845 immunotoxin, a fusion of de-bouganin and a humanized anti-EpCAM Fab antibody, did exhibit undesirable immunogenicity, but the antidrug antibodies were directed almost exclusively against the humanized Fab, as opposed to the de-bouganin toxin.[54••]

These examples support T cell epitope deletion as an effective strategy by which to suppress antidrug antibodies via upstream disruption of the immune response pathway. However, the above cited successes were derived in whole or in part from time-, labor-, and resource-intensive efforts that included: (i) epitope mapping via cellular immunoassays using large overlapping peptide panels spanning the full length of the protein, (ii) alanine scanning or similar mutagenic deletion of validated epitopes at the peptide level, followed by (iii) introduction of confirmed epitope-deleting mutations into the full length protein to assess structural and functional consequences, which are often unacceptably deleterious (Fig. 2A). Importantly, human class II MHC, composed of α/β heterodimers, are encoded by four different β-chain loci (DRB1, DRB3/4/5, DQB1, DPB1) and corresponding α-chain loci (DRA1, DQA1, and DPA1). Individuals may encode class II MHC from all four loci, and in general MHC II genes are highly polymorphic; nearly 3000 distinct alleles have been identified to date, though many of these alleles appear with low or extremely low frequency in global populations.[55] To better sample the genetic diversity of prospective patients, experimental epitope mapping often employs blood samples from 50 or more donors. Combined with the large number of overlapping peptides to be tested, the scale of such efforts can be both technically and financially imposing. More generally, the hit rate of epitope deleting yet function preserving mutations tends to be low in experimentally driven efforts (i.e., most tested mutations prove unacceptable), and thus more recent strategies have used computational methods to facilitate T cell epitope identification and deletion.

Computationally-driven T Cell Epitope Deletion

Computational T cell epitope predictors have proven reasonably accurate in benchmark studies.[56,57] While computational analysis enables rapid and facile prediction for large numbers of MHC alleles, extensive experimental studies have revealed a surprising degree of overlap in the peptide binding selectivities of various class II MHC proteins.[58] This in turn has enabled formulation of class II MHC supertypes, or groups of alleles that bind similar peptide repertoires.[59] Thus, the highly polymorphic nature of MHC II alleles can be

rendered more tractable by predicting for alleles that are both commonly encoded and broadly representative of key MHC II supertypes.[59,60] Additionally, experimental evidence suggests that immunodominant epitopes are those that bind multiple MHC II alleles, and therefore computational predictions can be further refined by searching for high risk “promiscuous” MHC II binders.[26,61] T cell epitope databases and prediction algorithms are regularly updated and improved,[62] and there exist codified strategies for employing these epitope predictors to guide immunogenicity risk assessment and protein deimmunization.[26,61] The next logical step is to fully integrate epitope predictors with computational protein design methodologies (Fig. 2B).

Conceptually, mutagenic T cell epitope deletion is a dual objective protein design problem: while deimmunizing mutations should disrupt class II MHC recognition and suppress downstream immune responses, each such mutation incurs a risk of compromising a protein’s native fold and therapeutic function. These two objectives, epitope deletion and maintenance of structure and function, are incommensurate and even competing in nature, and the goal of protein design is thus to identify variants that make beneficial trade-offs. Initial deimmunization algorithms employed simple BLOSUM substitution matrices or customized statistical sequence potentials in order to predict the structural and functional impacts of possible deimmunizing mutations.[63,64] More recently, structure-based design algorithms based on OSPREY[65] or Rosetta[66] have been described.[67-69] Pareto optimization approaches knit together both objectives in order to identify designs making optimal trade-offs: a Pareto optimal variant is not simultaneously dominated on both objectives by any other single variant, but instead improvement on one objective comes at the expense of the other.[70]

The P99 beta lactamase protein (P99βL) is a prospective component of Antibody Directed Enzyme Prodrug Therapy (ADEPT), and early work on deimmunizing the enzyme yielded minimally engineered 1- and 2-mutation variants.[50,71] More recent P99βL studies have sought to quantitatively assess the tradeoffs between immunoreactivity, measured by class II MHC binding of peptide fragments, and functionality, measured by thermostability and enzyme kinetics. An analysis of 4-mutation and 5-mutation designs showed that more aggressive sets of mutations were more disruptive of MHC II binding, but the eight engineered enzymes were found to have uniformly high catalytic efficiencies and melting temperatures such that clear tradeoffs were not readily observed.[72] Subsequently, a more expansive analysis was conducted with 18 deimmunized P99βL designs, bearing 1-8 mutations each, that comprised the Pareto frontier, i.e., *all* undominated designs. This more systematic analysis of the two-dimensional design space showed that the predicted immunoreactivity-functionality trade-offs mapped closely onto experimental observations, concluding that trade-offs are not only predictable but also designable.[73••] In a follow-on study, structure-based molecular modeling replaced the earlier statistical sequence potentials as a predictive measure of P99βL function. Structure-based design again confirmed that more aggressive sets of mutations were more disruptive of MHC II binding, and it yielded seven 8-mutation variants that all retained high level stability and activity (a 100% hit rate in this study).[74] In general, formulating T cell epitope deletion as a dual objective design problem has enabled a systematic and quantitative assessment of the immunoreactivity-functionality tradeoffs that are inherent to the deimmunization process.

In other studies a Rosetta structure-based deimmunization algorithm was used to delete murine MHC II restricted T cell epitopes from GFP. A 9-mutation variant was shown to have wild-type spectral properties, and murine T cells primed with wild-type GFP were significantly less reactive towards a mutant peptide constituting one of three epitope hotspots that had been targeted.[68•] The same algorithm was used to target three known epitopes in the PE toxin, yielding four single point mutants and one 2-mutation design. Three of the point mutants manifested wild-type or better cytotoxicity towards cancer cell lines, and peptide fragments of two were assessed for activation of human T cells and found to have lower overall immunostimulatory potential.[68•] These results further support computational protein design as a useful tool to guide biotherapeutic deimmunization.

Finally, structure-based protein design has enabled functional deimmunization of lysostaphin, a potent anti-MRSA bactericidal enzyme that suffers from undesirable immunogenicity.[75] Using a homology model, structure-guided re-design of lysostaphin's catalytic domain yielded a large number of 2- to 8-mutation Pareto optimal designs that retained high antibacterial activity, high thermostability, and yet suppressed molecular recognition by human MHC II proteins. Two candidates were shown to have wild-type *in vivo* efficacy yet significantly decreased *in vivo* immunogenic potential, as measured by immune cell proliferation in two different humanized mouse models (BLT mice and HLA transgenic mice).[76•] In a separate lysostaphin study, structure-based design algorithms enabled aggressive deletion of putative human HLA DRB1*0401 restricted T cell epitopes. Both individual optimal designs and combinatorial library designs were examined in these experiments, and both methods produced variants that strongly suppressed antidrug antibody responses upon immunization of humanized DR4 mice, which express the human DRB1*0401 HLA allele. Additional humanized mouse experiments demonstrated that the suppressed antidrug antibody response translated into enhanced therapeutic efficacy in a recurrent MRSA infection model.[77••] This latter result represents a milestone for the field: the first systematic and controlled demonstration that deletion of T cell epitopes not only reduces immunogenicity but as a result improves *in vivo* efficacy.

Discussion and Conclusion

Preclinical assessment of biotherapeutic immunogenicity is itself a complex and multifaceted problem, and the strengths and weaknesses of *in silico, in vitro*, and *in vivo* models have been reviewed recently elsewhere.[26,78] For instance, readers interested in the pros and cons of various transgenic and surgically engrafted humanized mouse models are referred to the following articles.[26,78-83] Ultimately, immunogenicity (or lack thereof) must be assessed in human subjects, but appropriate use of preclinical models can yield insights into immunogenic potential, as articulated in these review articles and the primary research articles cited above.

The upstream position of T cell epitope recognition in the immune response pathway suggests that mutagenesis of these subsequences might be particularly useful in designing immunoevasive biologics. In other cases, antibody epitope deletion could be critical for proteins to which patients have experienced prior natural exposures. Moreover, combining T cell and antibody epitope deletion strategies might represent a further step towards

comprehensive protein deimmunization. Indeed, published data on the T cell epitope depleted PE immunotoxin LMB-T18 suggests that a subset of T cell epitope deleting mutations fortuitously deleted antibody epitopes as well,[53••] and we have observed similar results with T cell epitope depleted lysostaphin variants (Griswold, unpublished data). More directed efforts to explicitly combine T cell and antibody epitope depleted designs are beginning to emerge, with analysis of the antibody and T cell epitope engineered immunotoxin LMB-T14 having published as the current manuscript was in revision.[84] This 10-mutation PE variant, which retained potent cytotoxic activity towards several cancer cell lines, was broadly evasive of both antibodies and T cells that recognized the wild type toxin. However, peptide fragments of the variant were found to activate T cell populations that predecessor molecules had effectively evaded, and it was subsequently found that mutations designed to delete antibody epitopes inadvertently introduced neo-epitopes recognized by prevalent class II MHC proteins. This work is indicative of the rapid advances being made in the deimmunized biologics space, but it also serves to highlight the remaining challenges and opportunities for further innovation.

With respect to such aggressive molecular engineering, it should be noted that the threshold for achieving “comprehensive” deimmunization remains unclear, as does the necessity of realizing this objective. What fraction of immunogenic epitopes must be deleted to deimmunize a protein? In a murine model study of interferon beta, deletion of one immunodominant T cell epitope did not result in a subsequent response against a subdominant epitope, [49] but in the case of the PE toxin it appears that targeting broadly distributed epitopes with higher mutational loads may be necessary.[53••] What fraction of patients exhibiting an immune response is acceptable? If a deimmunized biologic elicits a response in a significant fraction of patients, yet the response rate is half that of the wild-type predecessor drug, is that not a win? What of the strength of the immune response? Suppose a deimmunized biologic exhibits the same patient response rate as the wild-type protein but induces reduced titers of antidrug antibodies such that more patients may remain on the deimmunized variant longer. Is that an acceptable outcome? Can a highly immunogenic protein be redesigned so as to eliminate the response in all subjects? These questions hinge on a multitude of interdependent technical, clinical and financial factors, and definitive answers may remain elusive far into the future. In the near term, benchmarks for clinically useful deimmunization will likely be assessed on a case-by-case basis for each biotherapeutic and even specific patient populations. As experience with deimmunized biologics grows, perhaps more general rules for what it means to be deimmunized will emerge. In particular, deimmunized biobetters should provide rich data sets by which to probe the above questions in the context of existing clinical data for unmodified originator drugs.

There are a growing number of deimmunized biologics that are either moving towards or are currently in human trials. Long term, we expect that deimmunization technologies will have a profound impact on the biotherapeutics space. Key opportunities include both biobetter versions of FDA approved drugs with known immunogenicity issues as well as innovative new drugs whose therapeutic potential has yet to be tapped due to immunogenicity concerns. Analogous to the manner in which antibody humanization ushered in a wave of engineered

monoclonals, one might anticipate that the above deimmunization technologies will yield a revolution for non-immunoglobulin biotherapies.

Acknowledgements

We wish to generally acknowledge the dedicated community of researchers in the biotherapeutic immunogenicity space. Here, we have specifically highlighted recent work in which deimmunized biotherapeutics were characterized both functionally and immunologically, and we regret any inadvertent omissions and articles not cited due to space and thematic constraints. This work was supported by the National Institute of General Medical Sciences R01-GM-098977 and the National Institute of Allergy and Infectious Diseases 1R21AI098122 and 1R41AI118133. We also gratefully acknowledge computational resources provided by NSF grant CNS-1205521 and the technical support of the Dartmouth Transgenic and Genetic Construct Shared Resource (P30 CA023108) and Immunology COBRE Core C (P20 RR15639).

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P99βL. A large panel of 15 Pareto optimal deimmunized candidates (i.e., variants not simultaneously dominated on both objectives by any other single variant) was designed, constructed, and tested for immunoreactivity and molecular function. The experimental results mapped closely to the computed design space, thus demonstrating that tradeoffs in the deimmunization process are predictable and designable.

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Highlights

- Immunogenicity as a risk factor for biotherapeutic agents.
- Antibody epitope deletion as a strategy to evade antidrug antibodies.
- T cell epitope deletion as a strategy to silence the antidrug immune response.
- Multi-objective protein design algorithms to facilitate biotherapeutic deimmunization.
- Highlights of recent experimental validation for deimmunized biotherapeutics.

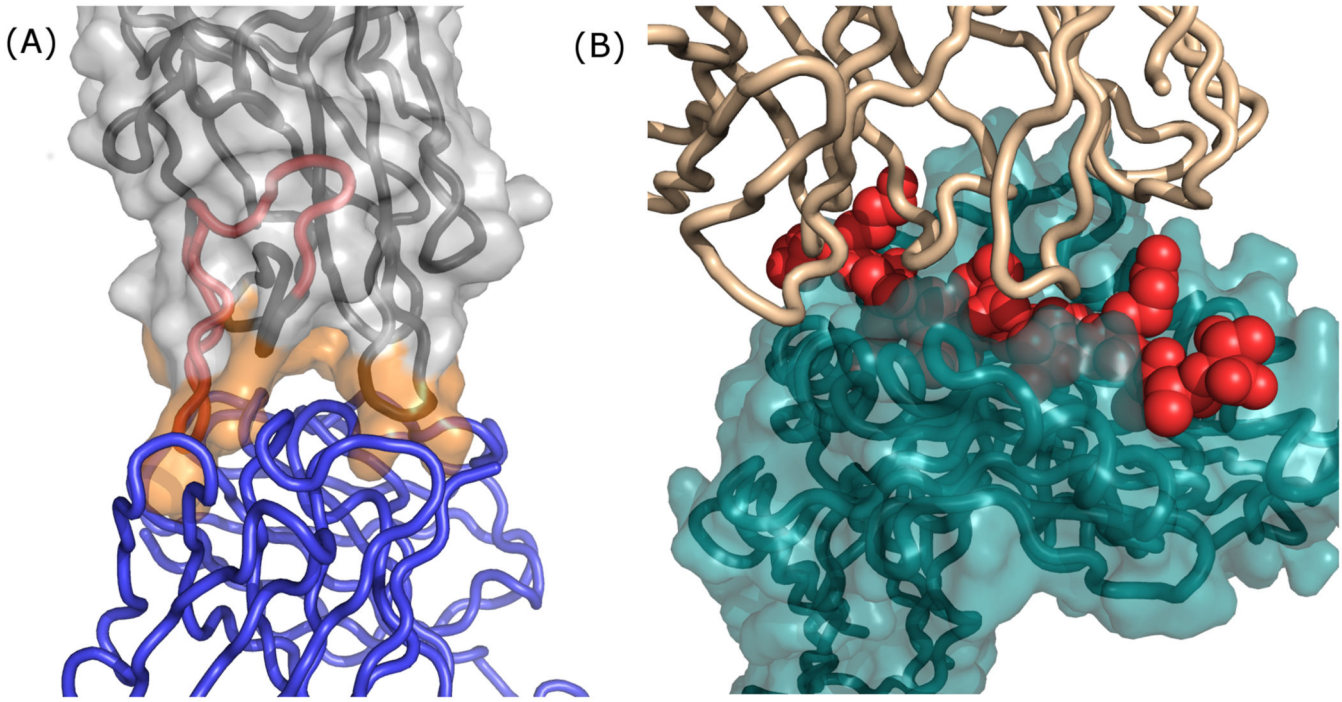

Figure 1.

Recognition and binding of antibody versus T cell epitopes occurs via separate molecular mechanisms. (A) A co-crystal structure of the factor VIII C2 domain in complex with an inhibitory antibody; PDB id 1IQD.[85] The inhibitory antibody is rendered as a blue polypeptide backbone, and the factor VIII C2 domain is rendered as a molecular surface with the underlying polypeptide backbone shown in grey. C2 domain surface residues at the antibody binding interface are colored orange. Independent of the C2 antibody epitope, an experimentally validated C2 T cell epitope (IEDB id 131093)[62] is highlighted as a red segment on the polypeptide backbone. Note that a protein's T cell epitopes may or may not overlap with its antibody epitopes. (B) Following proteolytic processing from internalized proteins, peptides that represent immunogenic T cell epitopes (red Van der Waals spheres) are bound in the cleft of class II MHC proteins (teal molecular surface with underlying teal peptide backbone); PDB id 1FYT.[86] The CDR regions of a cognate CD4+ T cell receptor are rendered as a tan polypeptide backbone. Formation of this ternary complex represents a key event that drives downstream development of high affinity antidrug antibodies. To reiterate, a T cell epitope need not share any amino acid residues with epitopes of resultant antidrug antibodies, though in this specific example there is at least some overlap. Images rendered with PyMOL (Schrodinger, LLC).

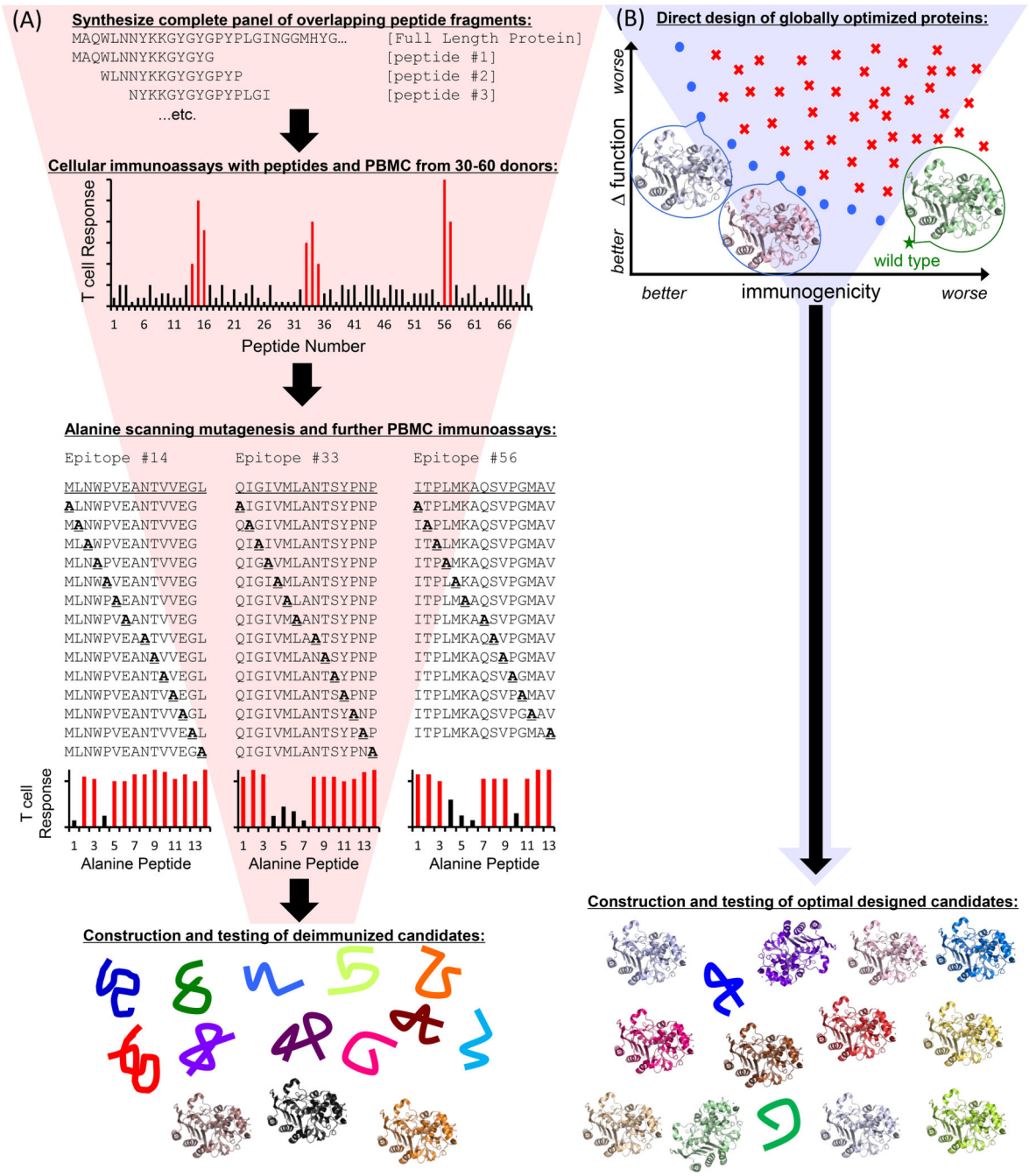

Figure 2.

Schematic diagrams for T cell epitope deletion strategies. (A) Experimentally driven deimmunization is a multistage process, moving top to bottom. A panel of overlapping synthetic peptide fragments spanning the full sequence is synthesized. The peptides are then tested for immune recognition, typically using *ex vivo* cellular immunoassays with blood cells from large panels of human donors. High responses to overlapping immunogenic peptides are indicated by tall red bars. Identified immunogenic peptides are subjected to alanine scanning mutagenesis and retested with the donor human immune cells. Alanine-

substituted peptides that reduce immune cell activation are highlighted as shorter black bars. Confirmed deimmunizing mutations are then engineered back into the full length protein and tested for expression, stability, and activity. Typical low hit rates are indicated by a majority of unfolded variant proteins, with only a few stable and active variants shown as cartoon structures. The process benefits from early identification of bona fide immunogenic peptides, but requires significant time and expense to funnel down to functional deimmunized candidates. (B) Computationally driven deimmunization addresses global protein design as a starting point. The protein design space is shown in two dimensions: predicted immunogenicity (x-axis) and predicted change in function (y-axis). Lower values are better in both objectives. Wild type has good molecular function but high immunogenicity. Sub-optimal designs are shown as red “x”es. The blue circles indicate Pareto optimal designs, or designs that are not simultaneously dominated on both objectives by any other single design. The Pareto frontier spans the full spectrum of optimal tradeoffs between the two objective functions. Representative protein designs are shown as cartoon structures. Individual, globally optimal designs balancing predicted reduction in immunogenicity and maintenance of function are selected for construction and analysis of expression, stability and activity. The high hit rate for folded and functional designs is indicated by a majority of cartoon protein structures. Computational optimization facilitates quick transition to validating candidates predicted to be functionally deimmunized. For both experimentally-driven and computationally-driven deimmunization projects, the final deimmunized candidates must be further tested for immunogenicity using cellular immunoassays and/or humanized murine models.