Monoclonal antibodies (mAbs) represent effective therapeutic agents as they demonstrate significant specificity for their targets and confer effector functions such as receptor-ligand blockade, target cell cytotoxicity and receptor antagonism. However, the use of mAbs in clinical settings has been complicated by a number of technical challenges including the demonstration of immunogenic responses. Immunogenic responses to antibody therapeutics can impact both safety and pharmacokinetic properties which can impact utility and efficacy of the drugs. A heightened awareness of these issues has developed as the field matures and the clinical consequences of immune responses to therapeutics have been reported and detailed. Understanding, controlling and engineering around potential immunogenicity is therefore of interest to the industry.

The use of antibodies as therapeutics has a long history. Prior to the development of mAb technologies, antisera from hyperimmunized animals were used to treat infectious diseases such as botulism and diphtheria. Diphtheria antitoxin, the antigen-specific IgG fraction isolated from the serum of diphtheria immunized horses, is still in use today. It is a life-saving therapeutic but is well known to cause significant immunological issues in patients, and is administered in a controlled setting where antihistamine is available for immediate application if needed. It is now obvious that injecting a person with a mixture of horse serum-derived proteins could cause immune reactions. It was less apparent that injecting patients with purified mouse-derived antibodies, i.e., murine mAbs, could also cause immune reactions. An appreciation of the consequences of an immune response to murine antibodies has lead to the development of engineered antibody constructs that carry a lower risk of immune reactions. Engineering of antibodies by sequentially replacing mouse sequence-derived amino acids for human sequences has in fact significantly reduced immunogenicity of this class of therapeutics. Chimeric antibodies were the first engineered improvement where the murine constant regions were replaced by human constant regions. The next development was the humanization process. Humanization results in an antibody where only the complementarity determining regions (CDRs) of the variable (V) regions are of mouse-sequence origin. The current state of the art is fully human amino acid sequence derived antibody
therapeutics where antigen specificity has been selected either in vivo by the use of genetically modified mice or by antibody engineering processes combined with screening.\textsuperscript{6-9} Fully human and humanized antibodies carry a lower risk for inducing immune responses in humans than mouse or chimeric antibodies.\textsuperscript{5}

It is fairly easy to characterize immune reactions such as immediate and delayed hypersensitivity responses. Antibody “inhibitor” responses that impact the efficacy of a protein therapeutic, such as those that develop in hemophilia patients treated with Factor VIII are also easy to characterize due to the severe clinical consequence of the immune response.\textsuperscript{10-12} The measurement of antibody responses directed at antibody therapeutics is more difficult to assess. These immune responses can be regarded as anti-idiotypic responses to the therapeutic when they are directed at the combining site, or as “binding antibodies” when they do not. While the development of hypersensitivity reactions is always of concern, the most often described consequence of the development of an immune response is neutralization of the therapeutic (anti-idiotypic response) and loss of efficacy due to modified pharmacokinetics (anti-idiotypic and binding antibodies; “anti-drug antibodies”). Low levels of transiently expressed anti-drug antibodies are not of significant concern as they rarely impact clinical outcomes.\textsuperscript{13} However, the development of high titer, high affinity anti-drug antibodies that interfere with the activity of the antibody therapeutic and in some cases associate with adverse effects are of concern and are the focus of this discussion.\textsuperscript{13-15}

\textbf{Causes of Immunogenicity}

There are many identified and purported causes of immunogenicity for antibody molecules.\textsuperscript{16-18} In order to minimize immunogenicity, it is recommended that as many of these factors are controlled as possible. Extrinsic factors such as aggregates and adjuvant-like contaminants are well known to cause issues and have been largely resolved by improvements in manufacturing and formulating practices.\textsuperscript{19,20} Extrinsic factors such as the co-medication of the patient, and the patient’s immunological status can also have profound effects on the reported immunogenicity of protein and antibody therapeutics.\textsuperscript{10,21} More subtle extrinsic factors that are difficult to eradicate are exemplified by the cytokine release syndrome induced upon administration of therapeutics such as anti-CD3.\textsuperscript{22} Interestingly, the immune response to a chimeric hamster-mouse anti-CD3 antibody in a mouse model of experimental autoimmune encephalomyelitis (EAE) can be reduced by controlling the extent of cytokine release with cyclosporine A.\textsuperscript{23} This result suggests that cytokine release is supporting a humoral immune response to the therapeutic by perhaps “short-circuiting” the adaptive helper T cell mediated response. Suppression of cytokine release may also partially explain the observation that co-medication with methotrexate reduces immune responses to antibodies and other protein therapeutics.\textsuperscript{24-26}

Intrinsic factors also influence the immunogenicity of antibodies. Antibodies directed at cell surface markers are deemed to have a higher risk of immunogenicity than antibodies against soluble factors. The reasons for this are not completely understood but may be due to antigen internalization and subsequent processing and presentation by target cells.\textsuperscript{27} Another intrinsic factor is the presence of carbohydrate side chains attached to the antibody via glycosylation sites conferred by the amino acid sequence of the light chain constant region, the heavy chain constant region or the V region itself.\textsuperscript{28,29} The presence of a galactose-alpha-1,3-galactose sugar within a carbohydrate structure on the Fab fragment of cetuximab was found to associate with severe anaphylactic reactions to the antibody. IgE antibodies specific for the galactose-alpha-1,3-galactose sugars were pre-existing in most of the donors, an uncontrollable extrinsic factor that contributed to the outcome.\textsuperscript{30} Notably, when cetuximab was manufactured in a cell line that could not add galactose-alpha-1,3-galactose to the antibody carbohydrate (Chinese hamster ovary derived manufacturing cell line), the resultant product was much less immunogenic.\textsuperscript{30} Other post-translational modifications to the antibody sequence may confer immunogenicity as well, such as glycation, deamidation and oxidation of amino acid side chains.\textsuperscript{31,32} Finally, the presence of CD4\textsuperscript{+} T helper cell epitopes have been described as correlating with immune responses to antibodies and other protein therapeutics.\textsuperscript{16,17,33-36}

The presence of IgG subclass antibodies during immune responses is typically associated with helper T cell activity.\textsuperscript{37} CD4\textsuperscript{+} T helper cells secrete cytokines that promote differentiation and isotype class switching by antigen-specific B cells. CD4\textsuperscript{+} T cells recognize linear sequential peptide fragments derived from the protein immunogen presented in the context of the donor’s HLA class II molecules. The activation, differentiation and migration of antigen-specific CD4\textsuperscript{+} T cells usually takes a few days after antigen exposure.\textsuperscript{38,39} This delay gives rise to the canonical “adaptive” antibody response profile characterized by the early temporal expression of IgM by antigen-specific B cells, with a subsequent isotype switching and affinity maturation event occurring once CD4\textsuperscript{+} T cells are activated. The presence of high affinity, IgG isotype, anti-idiotypic antibodies therefore indicates the activity of antibody-specific CD4\textsuperscript{+} T helper cells. Antibody V region specific CD4\textsuperscript{+} T helper responses have been well characterized in both mouse and human models,\textsuperscript{40-43} and are the aim of vaccines that utilize lymphoma idiotype proteins as tumor-specific antigens.\textsuperscript{44,45} It has not been possible to demonstrate CD4\textsuperscript{+} T cell specific for isologous constant regions.\textsuperscript{46} This result is likely due to the imprint of tolerance to the highly expressed constant region proteins. The exception is the ability to generate CD4\textsuperscript{+} T cell hybridomas to mouse constant region allotypes that represent novel immunoglobulin sequences in mouse strains that do not carry the identical allotype sequence.\textsuperscript{47} Antibody V regions, by contrast, contain CDRs that are unique to each B cell and are usually present in vanishingly small quantities. This is especially true of the somatically mutated CDR3 regions. The germline portions of V regions have not been demonstrated to induce immune responses in mouse models.\textsuperscript{40} Tolerance to novel V region sequences, as exemplified by somatically mutated CDR3 regions in particular, is incomplete or not present and V region specific CD4\textsuperscript{+} T cells are identifiable in the periphery.

CD4\textsuperscript{+} T cell responses include helper type responses that promote differentiation and activation of B cells and CD8\textsuperscript{+} cytotoxic cells (Th1, Th2 and T follicular helper cells), regulatory
responses that suppress B cell and T cell (Tregs) responses, and inflammatory Th17-type responses. Signaling strength of the T cell receptor epitope has some impact on the subsequent differentiation of CD4+ T cells, but the biggest effect is provided by the microenvironment where the antigen-specific CD4+ T cell encounters antigen. The microenvironment is impacted by the presence and type of adjuvant used as well as the route of antigen administration. The differentiation of CD4+ T cells to effector cells has been shown to be somewhat reversible in that the presence of certain cytokines can skew differentiation in cells that have already established a particular lineage. This result is of interest as patient populations differ by their immunological status; for example the chronic inflammatory status of autoimmune patients may have an impact on the overall immune responses to administered therapeutics.

**Antibody “Immunogenicity” may Reflect Homeostasis**

It seems counterintuitive that fully human sequence derived antibodies would cause immune responses in humans. Immunoglobulins occur at mg/ml quantities in serum and initiate expression very early in ontogeny, when tolerance to self proteins is imprinted on the nascent immune system. CD4+ T cells are tolerated or deleted in the thymus during development, and are anergized and deleted in the periphery upon contact with inappropriately expressed antigen. Multiple differential subsets of regulatory T cells control inappropriate responses to antigen in the periphery. B cells are tolerated by deletional and anergic mechanisms during development, but can also rescue themselves by a process of receptor editing. Receptor editing is the developmental process where B cells can rearrange their V region segments a second time, thereby altering specificity to avoid deletion. However, in spite of these mechanisms, human serum from non-diseased normal donors contains detectable levels of anti-idiotype antibody to a wide variety of autoantibodies. Anti-idiotype antibodies are akin to anti-drug antibodies against therapeutic mAbs, that is, they are antibodies with specificity for the unique V region of other immunoglobulin molecules. The presence of autoantibodies indicates that the tolerance system is not perfect and in fact, poly-reactivity to autoantigens re-emerges during the somatic hypermutation that takes place during an immune response. Anti-idiotype antibodies specific for the newly arising potentially problematic poly-reactive antibodies may represent an additional mechanism for assuring tolerance to self proteins. Indeed, for certain autoimmune diseases, these IgG anti-idiotype antibodies may be protective as the absence of an anti-idiotypic response correlates with the presence of disease in Type I diabetes. The presence of anti-idiotype antibodies is one proposed mechanism for the efficacy of IVIg in so many different autoimmune diseases. Therefore, mounting an antibody-mediated immune response directed at the combining sites of other antibodies likely is a normal, non-pathogenic event.

If antibody-specific CD4+ T helper cells can be activated in normal donors, even under steady-state conditions that lead to non-pathogenic, anti-autoimmune antibodies, then it should be no surprise that administering large quantities of antibody carrying a single specificity could induce anti-idiotype neutralizing IgG responses in some patients. This effect would be especially pronounced if the antibody happens to carry along a CD4+ helper T cell epitope presentable by the patients’ HLA class II molecules in its V region. These types of immune responses to therapeutic antibodies are of most concern as they tend not to diminish with time and can impact efficacy and associate with pharmacokinetic and safety issues. It is only when autoantibodies, or anti-therapeutic antibody responses, induce clinical sequelae that we pay attention.

**Fully Human Antibody Therapeutics can be Immunogenic**

Humanized antibodies contain murine-sequence derived CDR regions that have been engrafted, along with any necessary framework back-mutations, into human sequence-derived V regions. Fully human sequence derived antibodies have no murine sequence, and are largely produced via two sources: phage display technologies and transgenic mice. The first fully human sequence-derived antibody to be approved for therapeutic use was adalimumab (Humira), a fully human IgG1 antibody specific for TNFalpha that was selected via phage display of human VH and VL sequences. Recently, fully human sequence antibodies isolated from mice carrying genetic modifications such that the murine immunoglobulin genes were disabled and replaced with functional human immunoglobulin loci have been approved for therapeutic use. These antibodies undergo affinity maturation in vivo and therefore may represent a more naturally occurring set of sequences. The first of these, panitumumab (Vectibix), received approval for marketing in the US in 2006. Interestingly, even fully human sequence derived antibodies can induce marked immune responses. Adalimumab has been described as inducing neutralizing responses in a subset of patients that varies depending on the disease and the therapy (5–9% of patients with anti-panitumumab antibodies co-medicated with methotrexate, a total of 16% of patients displayed anti-drug antibodies at follow up.). Anti-drug antibody has been shown to correlate with a lack of efficacy in some adalimumab treated patients, meeting the generalized criteria for a significant immune response. Another fully human antibody that can induce a marked immune response in human patients is golimumab (Simpson), a fully human anti-TNF antibody derived from genetically modified mice. In rheumatoid arthritis patients co-medicated with methotrexate, a total of 16% of patients displayed anti-drug antibodies at follow up. The presence of anti-golimumab antibodies correlated with reduced trough levels of circulating antibody, a worrisome clinical event that may impact efficacy. On the other hand, panitumumab displays very low levels of anti-drug antibodies, in the range of 3–4%. The use of a Biacore type assay that can identify anti-drug antibodies with low affinities showed a 4% incidence. No clinical consequences have been reported due to the generation of anti-panitumumab antibodies, and anti-panitumumab antibodies only marginally affect pharmacokinetics. Within the subclass of currently approved fully human antibodies, therefore, the percentage of negligible, tolerable and marked immune responses is not clearly different from humanized antibodies (Table 1). This should not
be surprising given the human immune system's demonstrated ability to mount anti-idiotypic antibodies. It does suggest that fully human antibodies may have reached the limit of our ability to select for reduced immunogenicity antibodies without more directed engineering.

**Engineering Antibodies with a Reduced Potential for Inducing Anti-Drug Antibodies**

Many antibody therapeutics do not induce an immune response in human subjects. While it is difficult to compare products directly, it is clear that some antibody therapeutics do not induce measurable, clinically relevant immune responses. Therefore, it should be possible to engineer antibodies that carry a reduced intrinsic potential for inducing responses. Identifying and modifying CD4+ T cell epitopes present in antibody idiotypes may lead to the creation of antibody variants with a reduced immunogenic potential. There are many methods currently in use to identify CD4+ T cell epitopes in the amino acid sequence of proteins. Most of these methods are algorithms constructed based on HLA class II binding motifs that have been defined within identified peptide epitopes. As predictive methods tend to over-predict the number of functional epitopes in a given sequence, identified peptides must be subsequently tested for activity in vitro.

We chose to search for CD4+ T helper cell epitopes in the sequence of immunoglobulin V regions using a human cell based assay. This empirical method was selected due to the utility of the assay in identifying CD4+ T cell epitopes in other proteins with a very good specificity and sensitivity. This method identifies CD4+ T cell epitope responses within a test population of community donors. Proliferative responses to each peptide are compiled for approximately 100 donors, and peptides that induce proliferative responses in a statistically significant number of donors are identified. Community donors are presumed to not carry immunological memory to therapeutic proteins, therefore this assay identifies CD4+ T cell epitopes likely to induce immune responses upon initial contact with the protein.

Since VH and VL regions are small, they comprise a total of approximately 70 15-mer peptides overlapping by 12 amino acids. Prescreening for putative CD4+ T cell epitopes by readily available online predictive methods identified potential epitope sequences for subsequent testing scattered throughout the V regions. It was therefore expeditious to simply test all of the peptides at once.

Modification of CD4+ T cell epitopes can also follow algorithm-based methods. Again, we chose to select epitope peptide variants based on functional binding assays of our proposed variant antibodies, and then chose to test the variant peptides in a separate iteration of the cell based assay, as described for other protein therapeutics.

### Results

**Humanization can reduce immunogenicity by eliminating CD4+ epitopes in V region frameworks.** Humanization of antibody V regions has greatly reduced their immunogenic potential in vivo. Whereas approximately 40% of chimeric antibodies containing human sequence-derived constant regions and murine-sequence derived V regions induce marked anti-drug antibody responses in vivo, only 9% of humanized and antibodies containing human constant regions and human sequence-derived VH and VL framework regions do so. When V region peptides derived from the chimeric antibody cetuximab were tested for CD4+ T cell epitopes in vitro, a number of peptides induced prominent responses within our tested population (Fig. 1), consistent with the hypothesis that the presence of CD4+ T cell epitopes correlates with immunogenic potential. However, when the humanized equivalent V region was tested as peptides, there was an absence of prominent CD4+ T cell responses. The humanized version retained the exact mouse sequence-derived CDR sequences; only the framework regions were substituted. This in vitro example shows that the process of humanizing murine V regions can have a substantial impact on the immunogenic potential of an antibody. The responses to the peptides were segregated into either framework-sequence only peptides or CDR-containing peptides where at least one amino acid in the peptide is contributed by a Kabat defined CDR region. Further analysis showed that cetuximab amino acid-sequence framework peptides

### Table 1. Immune responses to fully human antibodies

| Target     | Indication         | Percent positive | Category (from ref. 5) | Reference |
|------------|--------------------|------------------|------------------------|-----------|
| panitumumab| EGFR               | 3–4%             | tolerable              | Label (http://www.vectibix.com/pdfs/misc/vectibix_pi.pdf); refs. 78, 80, 81 |
| ofatumumab | CD20               | 0%               | negligible             | Label (http://www.us.gsk.com/products/assets/us_arzerra.pdf) |
| golimumab  | TNFalpha           | 2–16%            | marked tolerable       | Label (http://www.simpconi.com/simpconi/assets/pdf/PrescribingInformation.pdf); ref. 78 |
| canakinumab| IL-1beta           | 0%               | negligible             | Label (http://www.pharma.us.novartis.com/product/pi/pdf/ilaris.pdf) |
| adalimumab | TNFalpha           | 5–12%            | tolerable marked       | Label (http://www.rxabbott.com/pdf/humira.pdf); refs. 74–76 |

www.landebioscience.com mAbs 259
were among those inducing prominent proliferative responses (Fig. 2). Responses to CDR sequence-containing peptides were also eliminated after humanization. The CDR containing sequences in cetuximab that induce the high responses contain contributions from murine frameworks, and when the similar but not identical sequences from the human frameworks used in the humanization were substituted, the overall response rate to the peptides was significantly reduced. CD4+ T cell proliferative responses to murine framework regions that are similar but not identical to human framework regions show that mouse antibody V regions are encountered by the human immune system as simply a protein immunogen, and that any tolerance induction that has occurred to human framework regions accords no special status to the mouse V regions.

CD4+ T cell epitopes occur only in CDR-containing regions of humanized antibody V regions. V regions of antibodies are composed of two (VL-JL) or three (VH-DH-JH) genomic segments. Gene segments are rearranged imprecisely, and additional trimming and nucleotide addition can occur at the joints. Imprecision in the joining mechanism is one contributor to the amino acid diversity of antibody CDR regions. In addition, somatic hypermutation during affinity maturation introduces amino acid variations. The purpose of all this variation is to select for high affinity, antigen-specific antibodies. On the other hand, the creation of novel amino acid sequences by these mechanisms can lead to immune responses as the immune system can not establish tolerance to every single new amino acid sequence randomly generated in this fashion. So once again, the creation of immunogenic epitopes within CDR regions is expected to occur with some frequency.

If our hypothesis is correct, CD4+ T cell epitopes should occur only in CDR region-containing peptides. CDR regions as CD4+ T cell epitopes is not a novel concept to individuals working on idiotypic vaccines for lymphomas. However, the idea that a therapeutic antibody may carry CD4+ T cell epitopes in the CDR regions has not been fully appreciated in the engineering of antibody therapeutics. To formally test our hypothesis and to determine the overall prevalence of CD4+ T cell epitopes in V regions, we assessed the CD4+ T cell epitope content of eight humanized and fully human therapeutic mAbs. Each set of immunoglobulin VH and VL region peptides was tested in vitro as 15-mer peptides in a human donor set of roughly 100 individuals per antibody. Two separate immunoglobulin VH and VL peptide sets were often analyzed together using peripheral blood cells from the same donor set. The datasets, once collected, were separated into germline-derived framework or CDR sequence-containing subsets as described above (Fig. 3A). We found a complete absence of prominent CD4+ T cell responses to the framework-derived peptides, consistent with previously published observations. The data shown represents 188 framework peptides from three different VH subfamilies (VH1, VH3 and VH4) and is compiled responses from a total of 793 donors. The absence of CD4+ T cell proliferative responses by the tested donors demonstrates the power of the human immune response to establish tolerance to framework regions from these VH and VL subfamily members. The prominent CD4+ T cell responses identified in the eight antibodies tested all contained CDR amino acid sequence contribution. The average response rate for framework peptides was lower than the response rate for the CDR region-containing peptides (Fig. 3B). The presence of CD4+ T cell epitopes only in the CDR region-containing peptides supports our hypothesis. Interestingly, even though the average response was increased for all CDR region-containing peptides, only a small subset induced prominent responses. For these eight tested antibodies, 2.5% (9 out of 364 tested peptides) of the peptides induced significant responses, with an average stimulation index of greater than 1.5 and average percent responses of greater than 10%. CDR sequence-containing epitope peptides were found in all three CDR regions of both VH and VL segments; no one CDR position was found to be more immunogenic among the eight antibodies tested. Responses to these epitope peptides tended to be associated with either numerous HLA class II alleles within our donor sets, or with widely expressed specific HLA DRB1 or HLA DQB1 class II alleles. The low number of CD4+ T cell epitopes found is consistent with the generally low immunogenicity of therapeutic antibodies, and also shows that CDR regions are not de facto immunogenic. That most CDR regions are not immunogenic suggests that immunogenic regions can be modified to remove this unwanted property; however, as the CDR regions confer affinity and specificity, the modification of these regions to reduce potential immunogenicity might be problematic.

Modification of CD4+ T cell epitope regions in antibody CDRs: is it possible? Most fully human and humanized antibodies are not notably immunogenic in their approved applications. When an antibody does induce significant levels of clinically relevant anti-drug antibodies, a likely culprit is a CD4+ T helper cell epitope. As we have shown, CD4+ T cell epitopes in antibody V
regions seem to occur only in CDR containing peptides. As most CDR regions are not overtly immunogenic, it should be possible to modify the epitope regions to reduce their immunogenic potential. The modification of CD4+ T cell epitopes within the V region of antibody molecules to produce a variant with the lowered immunological potential is termed “deimmunizing;” however, antibody CDR regions confer both affinity and specificity for antigen. Finally, modifications to amino acid sequences could potentially introduce de novo CD4+ T cell epitopes. To investigate whether deimmunization will be generally possible, we have characterized modified V region epitope peptides for three different antibodies in order to assess the potential for making an epitope response worse, better, or for having no impact on the activity of the epitope at all.

A total of five epitope sequences from the eight antibodies shown in Figure 3A were tested in the following way: the amino acid sequence of the epitope peptide was modified based on modeling and alanine scanning data (one epitope sequence) or based on guided selection of variants that were shown a priori to not impact affinity based on an empirical antigen-binding assay (four epitope sequences). A total of 202 peptide variants were tested in the in vitro proliferation assay. Note that changes in the epitope regions were designed to obviate impact on affinity only; no attempt was made to predict the critical HLA or T cell
response rate for the doubly modified peptides dropped to 57 ± 74%. We were especially surprised to see that for the double mutations, there were more variants with 10% or less of the control response rate (27% of all tested variants peptides) than there were for the single mutants (14%) without a concomitant increase in the numbers of “worse” epitopes: only 6% of all the doubly modified peptide variants displayed response rates over 200% of control as compared to 14% of the singly modified peptides. This suggests that two changes in a CDR are actually safer than making a single change.

For four of the modified epitope regions, changes were made to the sequence based on data showing that the particular point mutation had a minimal effect on affinity and bioactivity. Even so, when the double amino acid mutants were incorporated into the CDRs of full length antibodies only about 40% of the mutants retained full bioactivity. While the variants targeting the combining sites of these antibodies were selected for a lack of impact on affinity, it is of interest to note that incorporation of two random mutations into a protein sequence are predicted to result in an active protein in 41% of all cases. This number is derived from the demonstration that a single mutation in a protein sequence results in an inactive protein 36% of the time.

Discussion

Taken together, these results suggest that modification of CDR regions to eliminate or reduce the immunogenic activity of CD4+ T cell epitopes is possible. We were successful in the absence of any consideration for the HLA or TCR binding contacts within these epitope peptides. In the best of all circumstances, with two amino acid changes within a single CDR guided by selection of modifications that do not impact binding, a total of approximately 11% of all variants should be effective deimmunizing changes. We arrived at this conclusion by assuming that 40% of the effective double mutants within an epitope will retain full

![Figure 4. Reduction and exacerbation of in vitro immunogenicity by epitope amino acid sequence modifications. All proliferative responses rates to epitope variants were standardized to the unmodified parent epitope peptide response. (A) Distribution of standardized response rates of the variant peptides. (B) Distribution of standardized average stimulation index of the variant peptides.](image-url)
were resuspended in DMSO at approximately 2 mg/ml. After resuspension the peptide stocks were kept at -80°C.

CD4⁺ T cell proliferation assays. Peripheral blood mononuclear cell (PBMC) samples were drawn from community donors at the Stanford Blood Center (Palo Alto, CA). The CD4⁺ T cell proliferation assay was performed as described. 84,91 Briefly, dendritic cells were differentiated from human peripheral blood monocytes by culture in AIM V media (Invitrogen, Carlsbad, CA) plus GM-CSF and IL-4 for 5 days. Dendritic cells were activated by the addition of IL-1 and TNFα, and then collected for use on day 7. Autologous CD4⁺ T cells were isolated by negative selection from thawed PBMC samples (Stem Cell Technologies), and then co-cultured with irradiated dendritic cells and peptides (5 ug/ml) in AIM V media for 5 days. CD4⁺ T cells were used at 2 x 10⁵ cells per well, with 2 x 10⁴ dendritic cells as antigen-presenting cells. Proliferation was assessed on day 6. A donor was considered a responder for a specific peptide if the average proliferation of the experimental wells was 2.95 times higher than the control DMSO only wells. This cut-off value has been validated to return the highest sensitivity of positive versus false positive responses.84 Validation of the cut-off value was assessed by performing CD4⁺ T cell proliferation assays using peptides derived from a number of well-characterized protein antigens such as staphylokinase and HPV E6 and E7 and comparing the results to published T cell epitope mapping results. The positive control for the CD4⁺ T cell assay was tetanus toxoid included in 2–4 replicate wells at 1.25 ug/ml. The tetanus toxoid positive control must reach a stimulation index of at least 3.0 for the peptide proliferation results to be included in the database. All assay wells contained 0.25% DMSO, including the tetanus toxoid positive controls and the no-peptide negative controls.

**Materials and Methods**

**Peptides.** All peptides were manufactured as consecutive 15-mers overlapping by 12 amino acids describing the amino acid sequence of both the VH and the VL regions. Peptides were manufactured by Mimotopes (Melbourne, Australia). Peptides were pin synthesized, cleaved and lyophilized. Upon arrival, the peptides were resuspended in DMSO at approximately 2 mg/ml. After resuspension the peptide stocks were kept at -80°C.

- Figure 5. Two amino acid modifications in epitope sequences result in more potential deimmunized peptides. (A) Distribution of response rates to single point mutations. (B) Distribution of response rates to double mutations. (C) Box and whiskers plot standardized to the unmodified parent epitope peptide response. Arrow indicates the approximate location of the median.
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