

Peptide library-based evaluation of T-cell receptor breadth detects defects in global and regulatory activation in human immunologic diseases

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Edited* by William E. Paul, National Institutes of Health, Bethesda, MD, and approved April 9, 2013 (received for review February 4, 2013)

The ability of T-cells to respond to foreign antigens and to appropriately regulate this response is crucial for maintaining immune homeostasis. Using combinatorial peptide libraries, we functionally measured broad T-cell reactivity and observed impaired reactivity in established models of T-cell receptor repertoire restriction and in previously unrecognized disease contexts. By concurrently analyzing T-regulatory and T-effector cells, we show strong functional correlation between these subsets in healthy individuals and, strikingly, that alterations of this balance are associated with T helper type 2 (Th2)-mediated disease in a lymphopenic setting. Finally, we demonstrate that peptide-based priming of polyclonal naive cells with relatively low concentrations skews toward Th2 differentiation. These findings provide unique insight into the pathophysiology and functional consequences of abnormal T-cell repertoires and into differentiation of human naive T-cells.

atopy | immune deficiency

The ability of T-cells to respond to foreign antigens is crucial in mounting immune responses against viral, bacterial, and fungal pathogens and relies on the recognition of antigenic epitopes by T-cell receptors (TCRs). As the specific ligands that will be encountered are unknown, the immune system relies on maintaining a diverse TCR repertoire to ensure effective responses. “Holes,” or skewing of the repertoire, lead to missing responses and infection, as seen in HIV (1), aging (2) and various immune deficiencies (3), or abnormal responses leading to autoimmunity or atopy (4).

Global evaluation of T-cell reactivity is of fundamental importance for understanding disease etiologies and treating patients with suspected effector (Teff) or regulatory (Treg) T-cell repertoire abnormalities, but it is complicated by the need to assess the vast polyclonality of TCRs. MHC-tetramer technology has allowed for quantification of the number of epitope-specific CD8 or memory CD4 cells (5), and more recent advances using magnetic bead enrichment, synthetic antigens, and polyclonal amplification techniques have allowed for enhanced detection of rare, naive antigen-specific cells (6–8). However, examining selected peptides determined a priori precludes analysis of comprehensive TCR responsiveness. Well-established systematic approaches using synthetic-peptide combinatorial libraries have proven useful for identifying T-cell ligands including for transgenic mice (9) and T-cell clones (10, 11). In theory, use of similar libraries in a fundamentally different manner could be used to evaluate the reactivity of polyclonal T-cell populations to arrays of peptides. Such an application could be used to better identify and characterize diseases associated with T-cell repertoire restriction.

Repertoire restriction could affect the extent of overlap in antigen-specificity in reactivity between Teff and Treg repertoires. Dysregulation of such a balance could contribute to disease phenotypes. Considerable evidence indicates that Teff and Treg cells respond to the same exogenous antigens, presumably to fight infection while maintaining immune homeostasis (12–14). Studies in mouse models suggest that Treg TCR repertoire

breadth and the antigen-specificity of Tregs are important determinants in the suppression of autoimmunity, whereas restricted Treg TCR repertoires, specifically in the context of lymphopenia, can lead to T helper type 2 (Th2)-associated disease (15–17); however, analogous associations have not been identified in humans. Despite evidence for the intersecting reactivity of Tregs and Teffs, clonotypic analyses repeatedly suggest that even in the context of restricted TCR repertoires, the TCRs of Tregs are largely distinct from effector cells (18–22), complicating the recognition of abnormalities in regulatory cell specificity. Characterization of such diseases could have implications for treatment and for the role of Tregs in allergy and other forms of immune dysregulation.

We have used combinatorial peptide libraries to study the breadth of human polyclonal T-cell responses. By quantitatively evaluating responses of CD4 cells to a group of these libraries, we were able to discriminate normal and abnormal T-cell functional diversity in a variety of diseases. We also investigated the functional overlap between Treg and Teff reactivity and demonstrate that abnormalities in this overlap are associated with allergic disease in lymphopenic settings, providing evidence for an important role of regulatory cell antigen-specificity in immune surveillance. Finally, we examined human polyclonal CD4 differentiation from naive precursors and show that low peptide dose can skew toward Th2 differentiation.

Results

Peptide Pools Induce MHC Class II-Dependent Activation and Proliferation of CD4 Cells. To measure global functionality of T-cell subsets, we wanted a system capable of surveying reactivity to a broad array of antigens without particular concern for discerning individual activating epitopes. Previous studies have demonstrated the ability of soluble combinatorial peptide libraries to elicit proliferation of T-cell clones (9), and we speculated that they could be used to activate polyclonal T-cells. We synthesized pools of 15-mer peptides fixed at a single amino acid position and randomized at the other 14 positions (Table S1 and *SI Experimental Procedures*). The defined length of 15 amino acids optimized the system for stimulation of CD4 cells (23), and similar approaches in CD8 cells would be complicated by strict peptide length requirements (24). Twenty pools (1–20) were fixed with one of each of the 20 amino acids at position five, another 20 (21–40) at position six,

Author contributions: J.S.B., L.K.Y., and J.D.M. designed research; J.S.B. and L.K.Y. performed research; E.v.D. contributed new reagents; V.S., A.F.F., E.G., R.S., F.C., N.-p.W., and I.S. provided patient samples and clinical care; J.S.B. and L.K.Y. analyzed data; and J.S.B. and J.D.M. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1302103110/-DCSupplemental.

and another 20 (41–60) at position eight. Each of the nonfixed positions was synthesized with a random amino acid. Given the possibility for peptide ends to lie outside the open-ended MHC class II molecule (25), we chose central positions to fix to maximize the likelihood that those residues would lie within the binding groove.

Peripheral blood mononuclear cells (PBMCs) from a healthy donor were stimulated with individual peptide pools, and activation was measured by surface up-regulation of CD69 and CD98 on CD3+CD4+ cells after 40 h (Fig. 1A). The similar kinetics of CD69 and CD98 allowed for robust and consistent short-term measurement of activation. Individual pools induced up-regulation of these markers in a small but measurable percentage of cells (Fig. 2A). Similar activation was measured in naive or memory cells (Fig. 1B and Fig. S1A). The phenotypes of reactive cells were not significantly altered by stimulation, as sorted naive cells stimulated with these peptide pools in the presence of autologous dendritic cells (DCs) showed minimal changes in surface markers (Fig. S1B). Six-day stimulation (Fig. 1B) led to proliferation of peptide-specific cells, as measured by carboxyfluorescein succinimidyl ester (CFSE) (Fig. 2C). Stimulation was dose-dependent (Fig. S1E) and could be inhibited by an MHC class II blockade that did not reduce activation induced by anti-CD3 and anti-CD28 (Fig. 2D and E and Fig. S1C and D).

Aggregation of Responses to Multiple Peptide Pools Allows for Functional Analysis of CD4 T-Cell Repertoire. PBMCs were divided into 60 wells, each stimulated with a single peptide pool (Fig. 1A and B). In a single donor, the levels of CD69 and CD98 surface up-regulation and proliferation differed across peptide pools (Fig. 3A and Fig. S1F). We ranked the aggregate responses from largest to smallest, and responses were designated positive when the measured value was greater than four SDs above the mean of five unstimulated PBMC wells (Fig. 3B). Data from 11 healthy donors could be represented by mean and SE across peptide pools (Fig. S1G and H). Interestingly, compiled data revealed that certain fixed positions encouraged greater TCR binding across multiple donors, which could reflect public T-cell responses or differences in universal biochemical affinity for certain residues

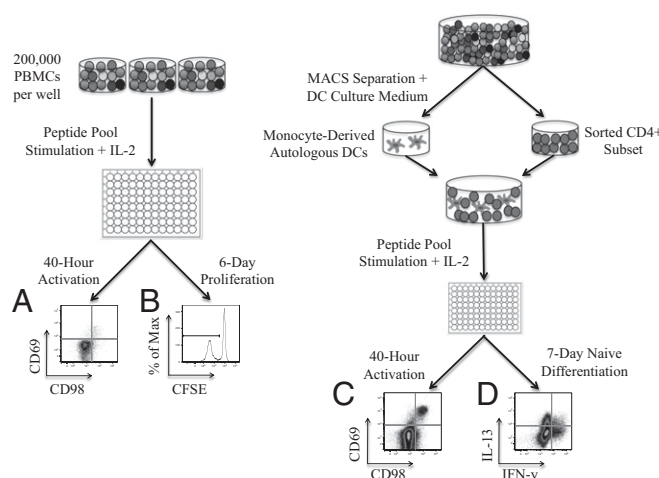


Fig. 1. Randomized peptide pools as a technique to evaluate TCR repertoire from PBMCs or sorted cell subsets. (A and B) PBMCs were cultured at 200,000 cells per well with IL-2 in multiple wells. (A) After 40 h, cells were stained for phenotypic markers and the activation markers CD69 and CD98. (B) Cells were stained with CFSE and cultured for 6 d. (C and D) Monocytes were isolated from PBMCs and cultured in IL-4 and GM-CSF. After 5 d, DCs were activated with TNF- α , and 2 d later were cocultured with autologous, sorted naive, or central memory CD4 cells in the presence of IL-2. (C) Forty-hour activation or (D) naive differentiation by intracellular cytokine stain.

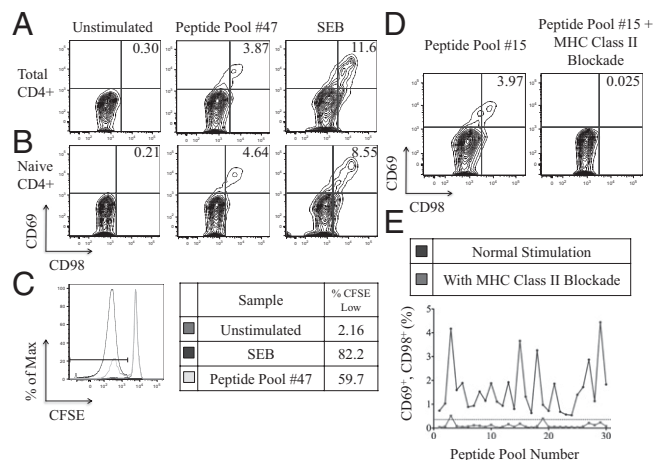


Fig. 2. Peptide pools induce MHC class II-dependent activation and proliferation of CD4 cells. (A–C) Shown are representative contour plots of CD69/CD98 up-regulation of gated total (A) or naive (B) CD4 cells from a healthy volunteer's PBMC in response to no stimulation, peptide pool #47, and staphylococcal enterotoxin B (SEB). (C) CFSE dilution of CD4 cells after 6 d after the same type of stimulation. (D and E) CD69/CD98 up-regulation of gated CD4 cells stimulated as in (A) in the presence or absence of an MHC class II blocking antibody added at time of stimulation. (D) Representative contour plots and (E) first 30 peptide pools. Dotted line represents mean percentage CD69/CD98 expression of five unstimulated wells.

(26, 27). As anticipated, across individuals, levels of short-term activation correlated with, but were not always accurate predictors of, proliferation (Fig. 3C). In addition, there was considerable overlap of reactivity between naive and memory subsets (Fig. 3D).

Although commonly used techniques for measuring TCR repertoires generally focus on numerical diversity, this method was instead focused on reactivity. We aimed to gauge the ability of an individual's T-cell compartment to respond to a broad spectrum of antigenic stimuli, which provides different information about TCR repertoires beyond whether the responding cells are clonally related or distinct. We therefore examined several models of altered TCR repertoires to measure the alteration's effect on functional diversity.

Previous studies have shown that naive cells have greater numerical TCR diversity than memory cells (28, 29). Across multiple healthy donors, naive CD4 T-cells appeared to have a more broad functional reactivity than memory cells, and collectively, naive cells responded positively to a mean of 89% and memory cells to a mean of 72% of peptide pools (Fig. 4A). It is possible that memory reactivity is underestimated, as memory cells had significantly higher background activation than naive cells, increasing the level of CD69/CD98 up-regulation required for a positive response. However, memory cells have been shown to have a decreased threshold of activation (30), which would be expected to increase the measured breadth of reactivity. Differences in background activation were not observed in any other comparisons we performed.

We next examined TCR reactivity across a large donor age range, as it has been reported that clonotypic TCR diversity of naive cells decreases with age (31). Functional naive diversity did indeed decrease with age, seemingly most markedly after the age of 80 y (Fig. 4B). However, in contrast to previous data using limiting dilution, we found that memory responsiveness was unchanged (Fig. S2A) (31). Background activation of naive cells or TCR reactivity of both subsets with anti-CD3 and anti-CD28 stimulation did not vary with age (Fig. S2B).

Analysis of CD4+ Repertoires Reveals Functional Repertoire Restriction in Human Immunologic Diseases. After validating the ability of our assay to detect T-cell repertoire alterations, we wanted to apply it

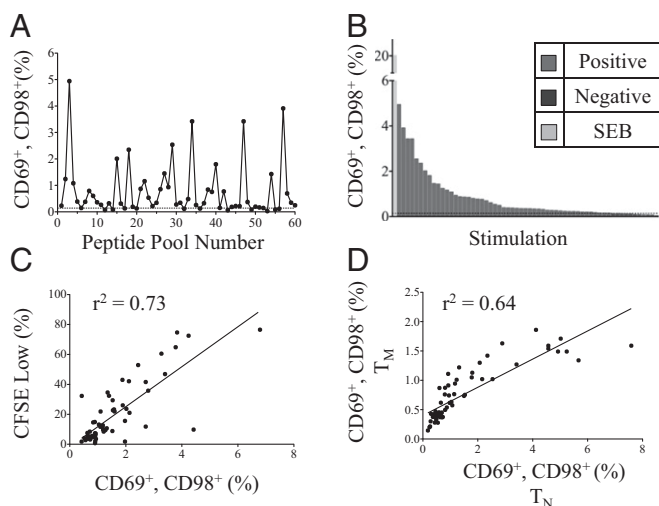


Fig. 3. Peptide pools differentially activate and induce proliferation of CD4 cells across normal controls. (A and B) Percentage CD69⁺ and CD98⁺ of CD4 cells from a representative control in response to 60 different peptide pools. Dotted line indicates four SDs above the mean of five unstimulated wells. (C) Correlation of activation and proliferation induced by peptide pools of a representative donor. The response to each peptide pool is denoted by a single dot. (D) Correlation between activation response of naive and memory CD4 subsets of a representative control.

to investigate repertoire restriction in immunologic diseases. We first analyzed samples from patients with idiopathic CD4 lymphopenia (ICL), a disorder with unknown etiology characterized by low CD4 counts and opportunistic infections (32) that had been reported to be associated with reduced TCR repertoire when measured by heteroduplex analysis (33). We found marked functional restriction of the naive TCR repertoire in patients with ICL, but not the memory compartment (Fig. 4 C and D and Fig. S2C), suggesting that the functional repertoire restriction is confined to this subset. Again, anti-CD3 and anti-CD28 stimulation and background activation were not different between patients and control participants (Fig. S2E). In addition, within the ICL cohort, the percentage of positive responses was not dependent on total CD4 counts (Fig. S2D). To further confirm that activated CD4 cells were independent of the overall percentage of CD4 cells, CD4 cells from a healthy donor were sorted, titrated back into the PBMCs at decreasing ratios, and stimulated with a peptide pool, which did not affect the surface activation levels (Fig. S2F).

We next examined a previously unstudied model of repertoire restriction by analyzing the reactivity of central memory CD4 cells from participants with STAT3 mutations leading to the autosomal dominant hyper-IgE syndrome (AD-HIES). Central memory cells from these patients have increased Ki-67 expression, consistent with high turnover compared with control patients (34). Such turnover either could be a result of compensatory homeostatic clonal expansion in response to the reduction of total central memory cells, which would lead to a reduction in the functional diversity of the cells, or could represent a differentiation state that immediately follows or precedes entry to or exit from this memory compartment, which should have no effect on diversity. Autologous monocyte-derived DCs were cocultured with sorted cell subsets, stimulated with peptide pools for 40 h, and stained for CD69 and CD98 surface expression (Fig. 1C). AD-HIES central memory cells responded positively to far fewer peptide pools than did control central memory cells (Fig. 4 E and F). This was not due to either an antigen-presentation or an intrinsic signaling defect because the frequency of peptide-responsive AD-HIES-naïve cells was similar to that seen in control patients, and the central memory cells up-regulated CD69 and CD98 appropriately after anti-CD3 and anti-CD28 stimulation (Fig. S3 A and B).

Inappropriate Functional Overlap Between Treg and Teff Subsets Is Associated with Atopy. Having demonstrated the potential of our peptide libraries to evaluate global TCR reactivity, we wished to analyze the relationship between regulatory and effector T-cell subsets simultaneously. By gating on Helios⁺ Foxp3⁺ CD4⁺ T-cells, we could concurrently interrogate TCR responsiveness of Tregs and Teffs (Fig. 5 A and B). In healthy donors, we found a strong and statistically significant correlation between Teff and Treg functional reactivity, indicating intersection of antigen reactivity (Fig. 5 C and E). Previously unactivated Tregs are most likely the source of these Foxp3⁺ Helios⁺ cells after activation, as similar activation of sorted CD25[−] CD127⁺ CD4 cells did not lead to significant Foxp3 or Helios up-regulation (Fig. S4A), and the small percentage of CD69⁺/CD98⁺ Tregs in unstimulated wells was similar to the total number of CD4 cells.

We then used our approach to determine whether a lack of functional correlation between Treg and Teff TCR repertoires might explain immune dysregulation seen in two models of human allergic disease associated with lymphopenia. Reconstitution of lymphopenic mice with a limited TCR repertoire can lead to elevated serum IgE and Th2 pathology, which appears to be dependent on the complexity of Treg TCRs (16). We therefore speculated that impaired functional overlap between Tregs and Teff could cause analogous, previously unreported allergic phenotypes defined by high IgE and atopy in a subset of patients with ICL (Table S2) or in a subset of patients treated for SCID resulting from adenosine deaminase deficiency (ADA-SCID) (Table S3) who also have high IgE and atopy.

We found that ICL patients with atopic phenotypes had markedly reduced correlation of Teff and Treg reactivity compared with patients with ICL without elevated IgE (Fig. 5 D and E) and that these results could not be accounted for by lower total CD4 cell counts or by cell signaling defects (Fig. S4 B and C). The presence of severe atopy alone does not necessarily lead to such Treg/Teff functional imbalances, as a patient without lymphopenia but with severe, uncontrolled atopic dermatitis had a normal Treg/Teff correlation (Fig. S4D).

Similar results were obtained from patients treated for ADA-SCID when comparing those patients with high IgE and specific allergic phenotypes with those patients without (Fig. 5E), even though similarly weakened TCR activation, elevated Th2 cytokines, and lymphopenia were noted in both atopic and nonatopic patients. Similar to the patients with ICL, these results suggest that the specific allergy noted in patients with ADA-SCID is associated with discordance between Treg and Teff reactivity.

Peptide Libraries Induce Differentiation of Naive CD4 Cells, and Low Peptide Dose Skews Toward Th2 Differentiation. In addition to the failure of Treg repertoires to adequately suppress Teff, the development specifically of Th2 disease in the context of lymphopenia has been postulated to be a result of the lack of high-affinity competition for given antigens (35). This is predicated on the assumption that lower-affinity T-cells would differentiate into Th2 cells because of weaker TCR signaling at priming (36). However, this phenomenon has not been directly observed in human cells because of the difficulty in identifying peptides that could prime sufficient numbers of polyclonal naive T-cells. Sorted naive, CFSE-labeled CD4 cells were cocultured with autologous monocyte-derived DCs and peptide pools (Fig. 1D). On day 7, cells were restimulated with phorbol-12-myristate-13-acetate/ionomycin and examined for cytokine production. Short-term activation, proliferation, and both Th1 and Th2 cytokines were observed with peptide pool priming in serum-free media with only exogenous IL-2 added (Fig. 6 A-C). Lower peptide doses led to a greater percentage of IL-13⁺ and IL-4⁺ cells among expanding cells and greater IL-4 transcript, as measured by real-time PCR, even with almost identical levels of proliferation at the higher doses (Fig. 6 D and E and Fig. S4E). Likely because of differences in stimulation, Th2-promoting doses differed between peptide pools and individuals; however, unlike IL-13 and IL-4, IFN- γ did not increase at lower concentrations (Fig. S5A).

respond to the peptide pools, such as whether their TCRs are related or distinct, or the types of peptides eliciting a response but, rather, to provide an unbiased view of global T-cell reactivity. Regardless of whether the cells responding to pools are clonally similar or diverse, a larger response to an individual pool or broader responses to many pools suggests an enhanced capacity to react to broad antigen repertoires.

Several limitations exist with most current measures of TCR repertoires; perhaps most important, that functionality is not directly measured but, rather, inferred from numerical measures. Sequencing of TCRs may provide the most direct measurement of diversity currently available, but technical constraints prevent analysis of heterodimer $\alpha\beta$ chains, thus omitting a significant source of functional TCR responsiveness (28). More important, primary TCR sequences do not confer knowledge of functional diversity: Single TCRs may be cross-reactive for multiple peptides, which can confer protection against infection or cause autoimmunity, and multiple TCRs can react to a single antigen (37–40). Further, the overlap in reactivity between T-cell subsets with distinct TCR sequences would go unrecognized using TCR sequencing alone. Our approach provides a direct method for measuring TCR functional diversity as it manifests in T-cell activation and proliferation and represents a complementary approach to TCR sequencing.

As recognized in previous studies, the observed age-associated decline in the naive repertoire has important implications for primary T-cell responses of older individuals (31). Analysis of the repertoires of patients with ICL suggests that they are able to effectively generate a diverse memory compartment, even though the functionality of their naive repertoire is compromised. We have also revealed restricted functionality of AD-HIES central memory repertoires, potentially contributing to the increased risk for varicella zoster virus (VZV) reactivation seen in these patients that was originally attributed solely to the reduction in number of central memory cells (34).

Reports investigating the intersection between Treg and Teff repertoires in mouse models have shown that TCR CDR3 sequences can overlap to a limited degree (19–21); however, distinct Treg and Teff TCR repertoires appear able to respond to the same foreign antigen (18, 41). Our data indicate that there is considerable correlation in reactivity between Treg and Teff subsets in normal control patients, implicating a mechanism for the control of inflammation in which effector and regulatory cells are activated by similar antigenic epitopes.

Although severe impairment of Treg activity leads to marked autoimmunity and allergic phenotypes in both humans and mouse

models (42, 43), it is unclear whether milder abnormalities such as the failure of antigen-specific Tregs to regulate effector cells specific for the same antigens could account for less severe clinical phenotypes. We show that reduced correlation of Treg and Teff reactivity to peptide pools in both patients with ICL and patients treated for ADA-SCID is associated with high IgE levels and allergy. A previously proposed mechanism for the development of Th2 pathology could account for this association: Studies in mice have found that restricted Treg repertoire combined with fewer total CD4 cells could lead to the emergence of low-affinity effector cells, which are normally suppressed by regulatory T-cells and concealed by high-affinity responses, responding to antigen (16, 35), and that these weak TCR interactions tend to favor Th2 differentiation, as previous studies have shown in mice (36) and as shown by data here in humans as well. Lymphopenia may exacerbate the potential for Treg/Teff discordance, and it is possible that similar dysregulation could exist in lymphoreplete settings.

The findings described here have many implications for both basic and clinical research. The restricted reactivity of the repertoires observed here in several contexts are likely to have functional consequences, such as increased infection in older individuals and patients with ICL, as well as increased susceptibility to VZV reactivation in patients with AD-HIES. Functional measurement of global Teff/Treg overlap may help predict and understand disease states such as autoimmunity or immune reconstitution inflammatory syndromes and graft vs. host disease. The significant correlation between activation of Treg and Teff subsets in normal control patients warrants further investigation into how this overlap manifests during antigen encounter, whereas the breakdown in this relationship underlying a Th2 phenotype suggests therapy targeting Treg specificity for the treatment of allergy. Wedded with TCR sequencing, measurement of functional overlap of activated Treg and Teff would permit direct interrogation of the relationship between the two repertoires. Furthermore, investigation of peptide-induced differentiation of naive cells may aid in the understanding of human polyclonal T-helper differentiation, which to date has only been studied with mitogens and superantigens.

Experimental Procedures

Subjects. Patients with AD-HIES were enrolled under National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board (IRB)-approved Protocol 00-I-0159 and/or Protocol 10-I-0148. Patients with ICL were enrolled under NIAID IRB-approved protocols 09-I-0069, 07-I-0033, or

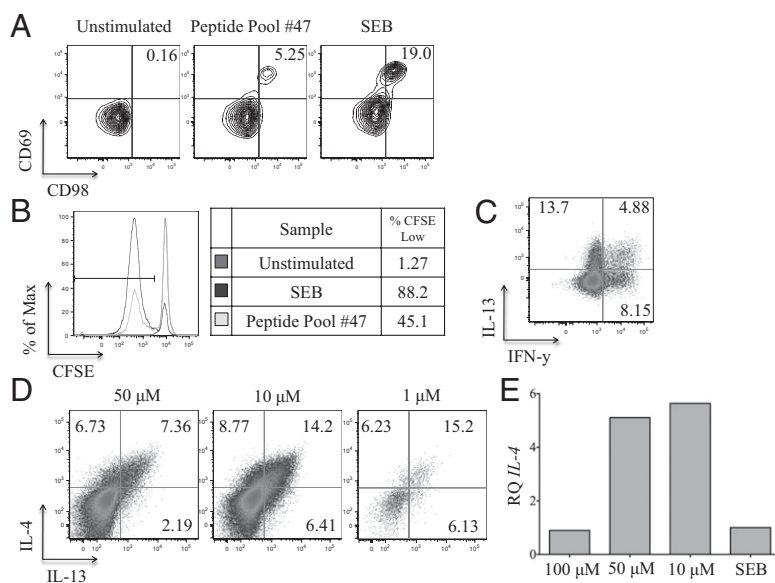


Fig. 6. Sorted naive CD4 T-cells activate, proliferate, and differentiate in response to peptide pools, and low peptide dose skews toward Th2 differentiation. Autologous, monocyte-derived DCs were cocultured with sorted or column-purified naive cells. (A) 40-h activation (B) 7-d proliferation (C and D) After 7-d stimulation with pool 47, cells were stained for Th1 and Th2 cytokines. Gated on CFSE low. (C) Th1 and Th2 differentiation (50 μ M used for stimulation). (D) Th2 differentiation across doses for peptide pool 18, representative of four independent experiments. (E) Real-time PCR analysis of IL-4 transcript across stimulant doses with peptide pool 57.

10-I-0148. Patients with ADA-SCID were enrolled under National Human Genome Research Institute IRB-approved Protocols 01-HG-0189 or 00-HG-0209. Three older patients were enrolled under Protocol GRC98-12-28-01. Buffy coats of normal controls were obtained from the National Institutes of Health Blood Bank. Peripheral blood mononuclear cells were prepared by Ficoll-gradient centrifugation and cryopreserved.

Stimulation Assays. PBMCs were thawed, washed with PBS, and resuspended at 1 million cells per milliliter in Roswell Park Memorial Institute medium 1640 (GIBCO) supplemented with 10% (vol/vol) FCS (Gemini Bio-Products), 100 U/mL penicillin, 100 µg/mL streptomycin, 29.2 µg/mL L-glutamine (all GIBCO) (R-10), and 5 U/mL recombinant human IL-2 (5 U/mL, PeproTech). To single wells in a 96-well plate, 200 µL of cell suspension were added such that each well contained 200,000 cells. For proliferation assays, the CellTrace CFSE Cell Proliferation Kit (Invitrogen) was used according to the manufacturer's protocol. Cells were stimulated with peptide pools (final concentration 100 µM, unless otherwise noted), staphylococcal enterotoxin B (1 µg/mL, Sigma-Aldrich), or anti-CD3 (OKT3; 1 µg/mL, eBioscience) and anti-CD28 (1 µg/mL, BD Biosciences). After 40 h or 6 d, cells were washed and analyzed by flow cytometry.

Coculture Assays. CD14⁺ monocytes were purified from PBMC by positive selection with antibody-coated microbeads (Miltenyi Biotec) and cultured in

R-10 with IL-4 (50 ng/mL, R&D Systems) and GM-CSF (100 ng/mL, PeproTech). After 5 d, TNF-α (10 ng/mL, R&D Systems) was added to activate DCs. On day 7, naive (aqua-CD3-CD4-CD25-CD27-CD45RO-) or central memory (aqua-CD3-CD4-CD25-CD27-CD45RO-) cells were purified by FACS sorting and cocultured at a ratio of 50:1 with autologous DCs in X-Vivo 15 serum-free media (Lonza). Cells were then cultured for 7 d before restimulation with phorbol-12-myristate-13-acetate (20 ng/mL, Calbiochem) and ionomycin (1 µM, Calbiochem) in the presence of Brefeldin A (10 µg/mL, Sigma) for 6–12 h. Intracellular cytokine staining was then performed.

Statistics. Statistical significance was calculated from the median value with a one-tailed Mann-Whitney test with GraphPad Prism 5.0 Software (GraphPad Software Inc), and r^2 values were calculated using GraphPad Prism 5.0 Software.

ACKNOWLEDGMENTS. We thank the members of the J.D.M. laboratory for their helpful discussions; the National Institute of Allergy and Infectious Diseases (NIAID) Flow Cytometry Core for assistance with cell sorting; all of the patients and clinical staff for invaluable contributions, C. Prussin for advice; and D. Douek, S. Boyd, and R. Germain for critical reading of the manuscript. The intramural research program of the NIAID supported this work.

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Supplemental Information

Barber et al. 10.1073/pnas.1302103110

SI Experimental Procedures

Peptide Libraries. Sixty peptide pools were synthesized by and purchased from Pepscan. The libraries were prepared on solid phase on a Syro multiple peptide synthesizer (MultiSynTech), using fluorenylmethoxycarbonyl/tert-butyl chemistry. The peptides were synthesized starting from a preloaded p-benzyloxybenzyl alcohol resin and, after completion, were cleaved from the resin with concomitant removal of the protecting groups. Pools comprised 15 mers fixed at a single amino acid position and randomized at all others. Twenty pools were fixed at position five, 20 at position six, and 20 at position eight. Each set of 20 had one pool fixed with every amino acid (Table S1). Four micromol of each library were resuspended in 200 μ L dimethyl sulfoxide (Sigma-Aldrich), followed by dilution with 200 μ L deionized water and stored at 4°C.

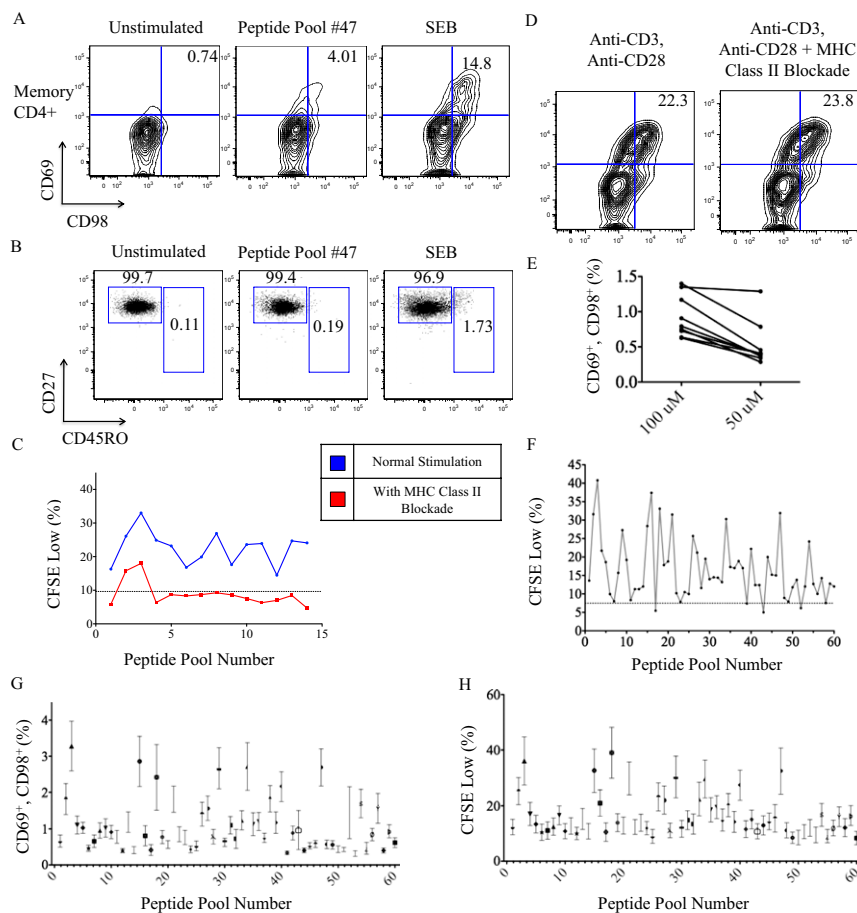
MHC Class II Antibody. IVA12 monoclonal antibody against HLA DR, DP, and DQ was purchased from American Type Culture Collection and provided by C. Prussin (National Institutes of Allergy and Infectious Diseases, Bethesda).

Allergic Diagnoses. Allergic diagnoses of patients with ADA-SCID and ICL were all made by the same team of allergists and immunologists on the basis of detailed clinical histories in conjunction with pulmonary function testing where relevant; skin prick testing (Greer Laboratories, Lenoir, NC); to 20 specific allergens (Army Centralized Allergen Extract Laboratory, Walter Reed National Medical Center,

Bethesda); and/or serum allergen-specific IgE levels performed by a commercial laboratory (Mayo Medical Laboratories, Rochester, MN) or by using the Phadia ImmunoCAP System (Phadia AB).

Flow Cytometry. Cultured cells were washed, thawed with PBS, and stained with aqua viability dye (Invitrogen). The following surface and intracellular markers were used for 40-h activation assays or for 6-d proliferation assays: CD3-QDot605 (Invitrogen); CD3-Alexa Fluor700, CD4-PE-Cy7, CD4-APC, CD8-APC-H7-PacificBlue, CD45RO-TRPE, CD27-PE-Cy5, CD69-FITC, CD98-PE (all BD Biosciences); and FoxP3-eFluor450 and Helios-Alexa Fluor647 (both eBioscience). The FoxP3 staining kit and protocol (eBioscience) was used to stain for both FoxP3 and Helios. Intracellular cytokine staining was performed using a saponin fix/perm protocol (Current Protocol in Immunology, unit 6.24). The following antibodies were used: IFN- γ -V450, IL-4-PE (both BD Biosciences), and IL-13-APC (BioLegend).

PCR Analysis. RNA was isolated with the RNeasy Mini Kit (Qiagen), as per manufacturer's instructions. cDNA was reverse transcribed with the High-Capacity Reverse Transcription Kit (Applied Biosystems). Triplicate real-time PCR reactions were performed in 20 μ L volume with 18s Hs99999901_s1 and IL-4 Hs00174122_m1 (Applied Biosystems). Transcripts were measured relative to 18S levels to normalize for cDNA inputs. PCR reactions underwent 40 cycles and were analyzed on an ABI 7500 Real Time Thermal Cycler (Applied Biosystems).



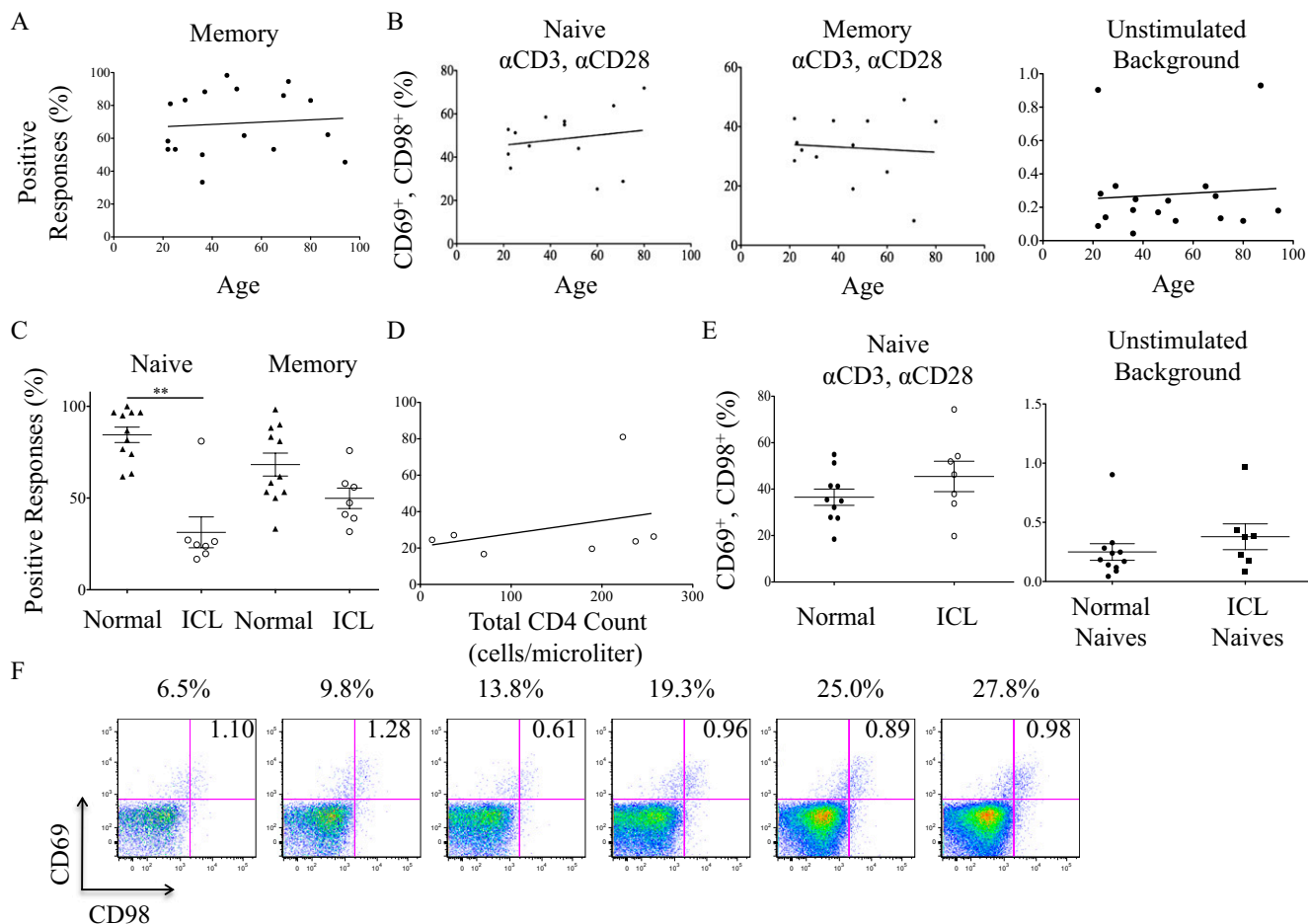


Fig. S2. (A) Percentage of positive responses to peptide pools of memory cells across ages. (B) Reactivity to anti-CD3 and anti-CD28 stimulation for naive and memory CD4 subsets, and background activation for naive cells. (C) Percentage of positive responses for naive and memory subsets for control patients ($n = 11$) and patients with ICL ($n = 7$) (** $P < 0.005$). (D) Total CD4 count plotted against the percentage of positive responses for ICL naive cells. (E) Anti-CD3 and anti-CD28 and background activation of naive cells. (F) CD4+ cells were sorted out from PBMCs and titrated back in at varying percentages, stimulated with peptide pool 26, and measured for CD69 and CD98.

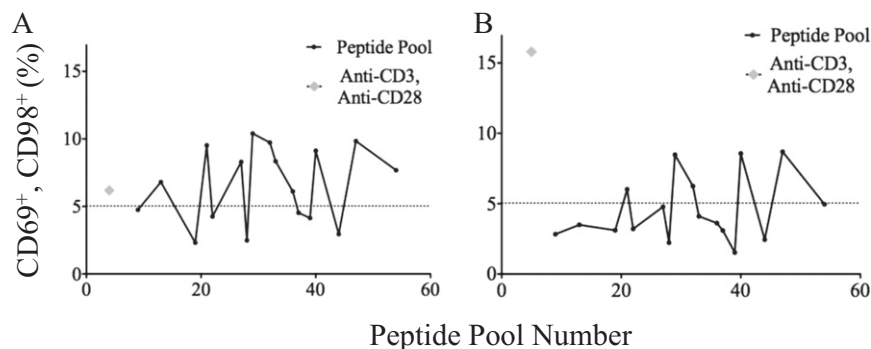


Fig. 53. (A and B) Autologous monocyte-derived dendritic cells were cocultured with sorted naive cells of a normal control patient (A) or a patient with AD-HIES (B). Shown are the activation responses to 16 peptide pools. Dotted line indicates 16 SDs above the mean of five unstimulated wells.

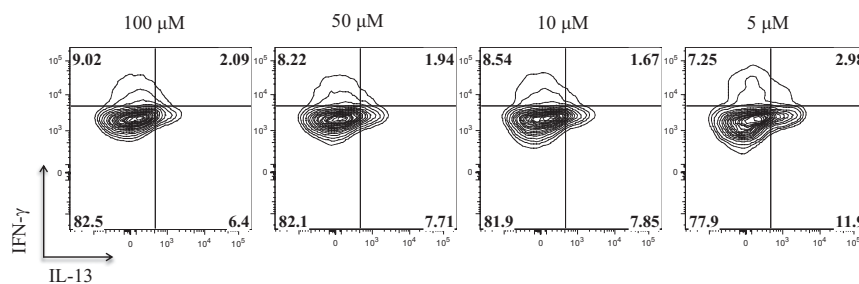
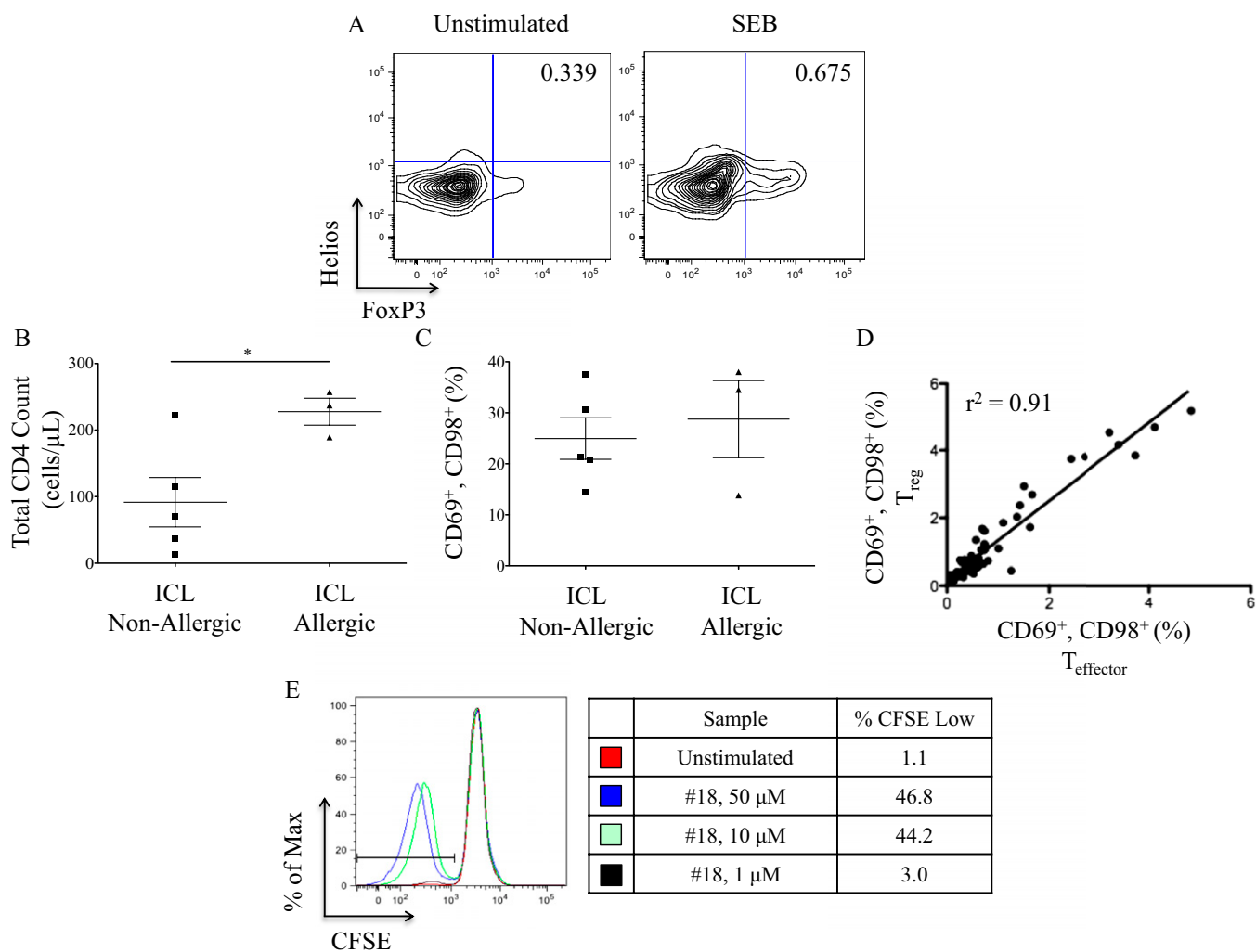


Table S1. Sixty randomized peptide pools

Pool no.	Peptides
1	XXXX A XXXXXXXXXX
2	XXXX C XXXXXXXXXX
3	XXXX D XXXXXXXXXX
4	XXXX E XXXXXXXXXX
5	XXXX F XXXXXXXXXX
6	XXXX G XXXXXXXXXX
7	XXXX H XXXXXXXXXX
8	XXXX I XXXXXXXXXX
9	XXXX K XXXXXXXXXX
10	XXXX L XXXXXXXXXX
11	XXXX M XXXXXXXXXX
12	XXXX N XXXXXXXXXX
13	XXXX P XXXXXXXXXX
14	XXXX Q XXXXXXXXXX
15	XXXX R XXXXXXXXXX
16	XXXX S XXXXXXXXXX
17	XXXX T XXXXXXXXXX
18	XXXX V XXXXXXXXXX
19	XXXX W XXXXXXXXXX
20	XXXX Y XXXXXXXXXX
21	XXXX A XXXXXXXXXX
22	XXXX C XXXXXXXXXX
23	XXXX D XXXXXXXXXX
24	XXXX E XXXXXXXXXX
25	XXXX F XXXXXXXXXX
26	XXXX G XXXXXXXXXX
27	XXXX H XXXXXXXXXX
28	XXXX I XXXXXXXXXX
29	XXXX K XXXXXXXXXX
30	XXXX L XXXXXXXXXX
31	XXXX M XXXXXXXXXX
32	XXXX N XXXXXXXXXX
33	XXXX P XXXXXXXXXX
34	XXXX Q XXXXXXXXXX
35	XXXX R XXXXXXXXXX
36	XXXX S XXXXXXXXXX
37	XXXX T XXXXXXXXXX
38	XXXX V XXXXXXXXXX
39	XXXX W XXXXXXXXXX
40	XXXX Y XXXXXXXXXX
41	XXXXXXXX A XXXXXXX
42	XXXXXXXX C XXXXXXX
43	XXXXXXXX D XXXXXXX
44	XXXXXXXX E XXXXXXX
45	XXXXXXXX F XXXXXXX
46	XXXXXXXX G XXXXXXX
47	XXXXXXXX H XXXXXXX
48	XXXXXXXX I XXXXXXX
49	XXXXXXXX K XXXXXXX
50	XXXXXXXX L XXXXXXX
51	XXXXXXXX M XXXXXXX
52	XXXXXXXX N XXXXXXX
53	XXXXXXXX P XXXXXXX
54	XXXXXXXX Q XXXXXXX
55	XXXXXXXX R XXXXXXX
56	XXXXXXXX S XXXXXXX
57	XXXXXXXX T XXXXXXX
58	XXXXXXXX V XXXXXXX
59	XXXXXXXX W XXXXXXX
60	XXXXXXXX Y XXXXXXX

Sixty pools of 15-mer peptides with a single amino acid position fixed and 15 randomized were synthesized. Pools 1–20 are fixed at position five, pools 21–40 at position six, and 41–60 at position eight. Random amino acid position is indicated by an X.

