C3d adjuvant effects are mediated through the activation of C3d-specific autoreactive T cells.

Anne S. De Groot[1,2], Ted M. Ross[3], Lauren Levitz[1], Timothy J. Messitt[1], Ryan Tassone[1], Christine M. Boyle[1], Amber J. Vincelli[1], Leonard Moise[1,2], William Martin[1], Paul M. Knopf[1].

[1]EpiVax, Inc., 146 Clifford Street, Providence, RI, USA; [2]Department of Cell and Molecular Biology, Institute for Immunology and Informatics, University of Rhode Island, 80 Washington Street, Providence, RI, USA; and [3]Vaccine & Gene Therapy Institute of Florida, 9801 SW Discovery Way, Port Saint Lucie, FL, USA.

Corresponding Author:
Anne S. De Groot, MD
EpiVax, Inc.
146 Clifford St.
Providence, RI 02903
Telephone: (401) 272-2123
Facsimile: (401) 272-7562
AnnieD@epivax.com

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ABSTRACT

Complement fragment C3d covalently attached to antigens enhances immune responses, particularly for antigens lacking T cell epitopes. Enhancement has been attributed to receptor cross-linking between complement receptor CR2 (CD21) and polysaccharide antigen to surface IgM on naïve B cells. Paradoxically, C3d has still been shown to increase immune responses in CD21 KO mice, suggesting that an auxiliary activation pathway exists. In prior studies, we demonstrated the CD21-independent C3d adjuvant effect might be due to T cell recognition of C3d T helper epitopes processed and presented by MHC class II on the B cell surface. C3d peptide sequences containing concentrated clusters of putative human C3 T cell epitopes were identified using the epitope-mapping algorithm, EpiMatrix. These peptide sequences were synthesized and shown *in vitro* to bind multiple human leukocyte antigen (HLA)-DR alleles with high affinity, and induce IFNγ responses in healthy donor PBMCs. In the present studies, we establish further correlations between HLA binding and HLA-specific lymphocyte reactions with select epitope clusters. Additionally, we show that the T cell phenotype of C3d-specific reactive T cells is CD4+CD45RO+ memory T cells. Finally, mutation of a single T cell epitope residing within the P28 peptide segment of C3d resulted in significantly diminished adjuvant activity in BALB/c mice. Collectively, these studies support the hypothesis that the paradoxical enhancement of immune responses by C3d in the absence of CD21 is due to internalization and processing of C3d into peptides that activate autoreactive CD4+ T helper cells in the context of HLA class II.

Key words: Autoimmunity, C3d, Complement, T cell epitope, Vaccine

INTRODUCTION

Two methods of adjuvant conjugation to antigen have been shown to greatly improve the efficacy of polysaccharide vaccines: use of an immunogenic foreign T cell epitope-rich protein, and use of complement fragment C3d.[1–3] The adjuvant effect of C3d is said to result from the synergistic effect of two events: (1) polysaccharide antigen binding to the IgM/D B cell receptor, and (2) C3d binding to the B cell complement receptor 2 (CR2 or CD21). The co-activation of these two signals has been shown to lower the threshold of activation and to promote a more robust immune response by inducing isotype switching, somatic hypermutation, and B cell memory generation.[4] Yet in mice lacking complement receptors CR1 (CD35) and CR2 (CD21), immune responses to protein antigen-C3d conjugates were equally enhanced to the levels seen in wild-type mice.[5–7] Further, C3-/- mice, but not CD21-/- have been shown to be deficient in T-dependent (Td) responses.[8] These findings suggest that the current model for C3d activity may be incomplete and an auxiliary immune activation pathway exists.

We have hypothesized that in addition to its molecular adjuvant property enhancing signal 1 in antigen-specific B cell activation, C3d also contains T cell epitopes that stimulate autoreactive C3d peptide-specific T helper cells, creating a response similar to that of any cognate T helper epitope.[9] Multiple repeats of polysaccharide residues can cause cross-linking of antibody regions and internalization of the conjugate into the B cell antigen processing compartments. Processing and presentation of any protein components associated with the polysaccharide antigen that bind to MHC will ensue: the resulting peptide-associated MHC II complexes presented on the B cell surface generate help by activating CD4+ T cells via recognition of T cell antigen receptors. This induction of Td responses may result in the generation of T cell help for any anti-polysaccharide B cell responses, generating higher antibody titers and higher affinity antibodies than T-independent (Ti) responses.

In previous studies, we used the EpiMatrix algorithm to identify T cell epitopes in complement C3 and assessed their binding potential to multiple HLA haplotypes.[9] We determined that the frequency of putative T cell epitopes in complement C3 was greater than many other human plasma proteins analyzed using the EpiMatrix algorithm. Further, the distribution of these putative T helper epitopes within C3 was non-uniform and concentrated within the C3d segment. Thus, we have postulated that the previously-observed enhancement of the immune response by C3 might be due in part to the activation of C3d peptide-specific autoreactive T helper cells. This discovery of T cell epitopes within C3d offers a novel explanation for the

adjuvant effect of C3d and a potential resolution of the paradoxical results observed in CD21 KO mice.

Here, we examine three putative T cell epitope "clusters" from C3d that were identified in earlier studies.[9] *In vitro* HLA binding studies and *ex vivo* PBMC responses from four separate HLA-typed donors correlated with epitope mapping predictions. Further, *ex vivo* PBMC responses to C3d peptides were found to be associated with a CD4+CD45RO+ population of memory T cells, suggesting that C3d-specific T cells are autoreactive memory T cells. Finally, the contribution of T cell epitope-mediated immunogenicity to C3d activity was confirmed by mutation of the single predicted T cell epitope in the P28 peptide segment of C3d expected to reduce binding to murine MHC. In immunization studies, T cell responses to hapten-conjugated C3d mutants were diminished, further supporting the contribution of autoreactive T helper responses to the adjuvant activity of the C3d fragment of C3.

RESULTS

Epitope analysis and peptide synthesis

Candidate T cell epitopes in C3 were identified using the EpiMatrix algorithm as described in Methods and reference 9. Within the 302-amino acid C3d sequence, 11 putative T cell epitope clusters with significant cluster scores[10] were identified. Three peptides (aa 32-50, 100-118, and 190-209) were excluded from further study, as they had three of the four lowest cluster scores; two additional peptides (aa 73-96 and 176-198) were not selected for synthesis due to high hydrophobicity. The informatics analysis was repeated on the remaining six putative epitopes in 2010 with a revised version of the EpiMatrix algorithm, which had been retrained on a more robust set of epitopes using the expanded set of sequences available in 2009. This updated matrix is improved relative to the 2002 matrix and has demonstrated greater accuracy when benchmarked against other prediction tools.[11,12] From the six putative epitopes previously identified, three peptides: $C3d_{223-246}$, $C3d_{248-265}$, and $C3d_{269-286}$ were selected for further study based on their updated EpiMatrix scores, broad binding to a range of HLA in competition binding assays, and ability to induce IFNγ responses in non-HLA typed donors (see ref. 9 and **Table 1**). All three clusters are distributed in Region 2 (aa 175-290) of C3d; notably, $C3d_{223-246}$ overlaps with the C3d peptide fragment P28 (aa 208-235), identified by Lambris et al.,[13] by 13 amino acids (**Figure 1**).

HLA binding validates *in silico* predictions

Class II HLA binding assays were conducted to validate the *in silico* EpiMatrix predictions, as described in Methods. Each of the C3d epitope peptides bound to at least one of the six different HLA DR alleles tested with high affinity (see **Table 2**). Peptide $C3d_{223-246}$ bound with high affinity to four alleles (0101, 0401, 0701, 1101), moderate affinity to one allele (0301), and weak affinity to one allele (1501). Peptide $C3d_{269-286}$ bound with high affinity to four alleles (0101, 0401, 0701, 1101) and weak affinity to two alleles (0301, 1501). Peptide $C3d_{248-265}$ bound with high affinity to one allele (1101) and did not bind to the other five alleles tested. For greater than two-thirds of the peptide/HLA allele combinations, binding results confirmed EpiMatrix predictions (**Table 2**). Comparison of HLA binding results to our earlier work reveals some discrepancies with those found in current studies due to a number of modifications to the assay to improve robustness and sensitivity over time. This includes the use of peptides capped at their N- and C-termini to improve peptide stability, which likely contributed to improved binding affinity among predicted peptides that were non-binders in our earlier work (e.g., peptides $C3d_{223-246}$ and $C3d_{269-286}$ had high cluster scores but either did not bind or bound only weakly to 0101 and 0701 in the previous study, but both peptides had improved binding affinity to these alleles once capped). Furthermore, optimizations were introduced to increase signal-to-noise ratios; as a result, peptides that had previously bound weakly, possibly due to an initial high background producing a false positive signal (e.g. $C3d_{248-265}$ binding with high affinity to 1501 in the previous work), exhibited weak or no detectable binding in the improved assay. Finally, the use of an updated, more accurate EpiMatrix algorithm in the current study led some peptides that were previously predicted to bind given HLA alleles to be re-classified as non-binders (e.g., $C3d_{248-265}$ was originally predicted to bind 1501, but its updated EpiMatrix score classifies it as a non-binder for this allele, which corresponds to the negative binding results *in vitro*). Comparison of the results in this work to previous studies reveals improved correlations between *in silico* predictions and *in vitro* binding (83% correlation in this work vs. 50% correlation in earlier work), which we attribute to these assay improvements and use of the improved EpiMatrix algorithm.

C3d epitopes induce IFNγ responses from multiple donors

To confirm observations from HLA binding assays and correlate C3d-specific T cell responses to HLA background, we stimulated PBMCs from HLA-typed donors and tested for cytokine responses to C3d peptides by IFNγ ELISpot. PBMCs from four healthy donors were stimulated with a C3d peptide pool (comprised of $C3d_{223-246}$, $C3d_{248-265}$ and $C3d_{269-286}$) or an irrelevant

peptide (Pan-DR epitope; PADRE) for 10 days to expand the number of C3d-specific T cells from baseline. Harvested PBMCs were then re-stimulated with the C3d peptide pool, individual C3d peptides, PADRE, phytohaemagglutinin (PHA, positive control), or no peptide. ELISpots were used to determine IFNγ secretion two days after re-stimulation. Each plot in **Figure 2** shows IFNγ responses from one of four donors to each stimulation condition. As shown in **Figure 2**, most of the donors responded specifically to at least one C3d peptide, and all donors responded to the C3d peptide pool. No response was seen to these same peptides when donor cells were initially cultured with PADRE and then restimulated with C3d peptides. In most cases, ELISpot responses correlate well with EpiMatrix predictions and HLA binding. For example, Subject 666 (HLA DRB1*0701) responded strongly to $C3d_{223-246}$ and $C3d_{269-286}$ as predicted by EpiMatrix and as observed by HLA binding assay specifically for this donor's HLA allotype (see **Table 2**, **Figure 2A**). Additionally, a response was observed for all three C3d peptides, including $C3d_{248-265}$, for Subject 705. The HLA type of this donor (HLA DRB1*1101) matches the only allele predicted and observed to bind this peptide in HLA binding assays (**Table 2, Figure 2C**). Subject 703, with HLA DRB1*0301 (**Figure 2D**), generated a low response to $C3d_{223-246}$ and to the C3d peptide pool. Again, this result corresponds to the binding data; HLA DRB1*0301 only moderately bound $C3d_{223-246}$, and only weakly bound or did not bind $C3d_{269-286}$ and $C3d_{248-265}$, respectively (**Table 2**). The only discordance observed with HLA binding was in Subject 706 (HLA DRB1*0401), who responded strongly to $C3d_{223-246}$ as predicted and observed for HLA binding, but did not respond significantly to $C3d_{269-286}$ (**Table 2**, **Figure 2B**).

Responses to C3d peptides are generated by CD4+ memory T cells in the periphery

In our previous work, we used immunophenotyping and fluorescence-activated cell sorting (FACS) to demonstrate that the IFNγ response to C3d peptides was primarily generated by CD4+ cells.[9] To extend this observation and confirm that C3d-specific CD4+ T cells also express cell surface markers characteristic of memory T cells (providing support for our hypothesis that C3d-specific memory T cells circulate in the periphery), we repeated the FACS assay with additional immunophenotyping to determine whether the C3d-specific response was due to naïve or memory T cells. A representative experiment is shown in **Figure 3**, in which PBMCs stimulated with a C3d peptide pool at a total concentration of 30 μg/ml or with MOG_{35-55} (negative control peptide) at 30 μg/ml were cultured for 10 days. Similar to C3d epitopes, MOG_{35-55} is an autologous T cell epitope that binds multiple HLA types.[14] While MOG_{35-55} is an auto-antigen in multiple sclerosis, the peptide does not induce an immune response in healthy individuals. On day 10, PBMCs were stimulated with the C3d peptide pool, MOG_{35-55}, the

polyclonal stimulator CytoStim (Miltenyi), or media alone for three hours. After three hours of secondary stimulation, IFNγ-positive cells were enriched using an IFNγ secretion assay kit (Miltenyi), stained with antibodies for CD4, CD45RA (naïve marker) and CD45RO (memory marker), and analyzed by flow cytometry. We gated first on CD4+, IFNγ+ cells and then on the CD45RA and CD45RO populations.

As shown in **Figure 3**, memory (CD4+IFNγ+CD45RO+) T cells are the dominant population responding to the C3d peptides in this assay system. A mixed naïve (CD4+ IFNγ+CD45RA+) and memory (CD4+IFNγ+CD45RO+) response was generated to CytoStim, indicating naïve T cells could be re-stimulated under these conditions. This is in contrast to the negative control peptide, MOG_{35-55}, which did not induce an autoreactive memory (or naïve) T cell response. These results suggest that T cell responses are specific to C3d, are not seen in response to other autologous peptides (in healthy control subjects), and are mediated by autoreactive memory T cells circulating in the periphery.

Disruption of the single T cell epitope in P28 diminishes the activity of C3d *in vivo*

It has been proposed that the adjuvant effect of C3d involves interaction between BCR:polysaccharide and C3d:CD21 complexes, lowering the threshold of B cell activation by engaging CD19 with Igα/β.[15,16] While the P28 peptide segment (amino acids 208-235 of C3d) has previously been implicated as mediating C3d binding to CD21[13,17,18], this role has been disputed based on the 2011 crystal structure of C3d interacting with CD21.[19] Rather, the finding that the P28 segment harbors a T cell epitope provides support for the reported adjuvant properties of P28, whether or not it is involved in mediating binding to CD21. The adjuvant activity of multiple copies of P28 has been established in the context of protein immunogens[20] and DNA vaccine immunogens.[5] Indeed, a 4X repeat of the P28 peptide segment retains equal adjuvant activity to a 3X repeat of full-length C3d when delivered as a recombinant DNA vaccine with the HIV-1 ENV_{gp120} glycoprotein.[5] An EpiMatrix analysis of the P28 peptide segment revealed a single promiscuous HLA binding motif located at the C-terminus of P28 (**Figure 4**). Interestingly, this single T cell epitope overlaps with the N-terminal T cell epitope in the $C3d_{223-246}$ epitope cluster described in this work, which is shown to bind multiple HLA alleles *in vitro* and to induce IFNγ responses in multiple donors (**Table 2**, **Figures 1** and **2**). We hypothesized that if the adjuvant activity of P28 is abolished or diminished due to mutation of this single T cell epitope, it would help explain the adjuvant activity of P28.

Using EpiMatrix, we selected two variants of the T cell epitope in the P28 peptide segment which were designed to disrupt HLA binding to eight of the most common HLA types (covering >90% of human populations) and mouse MHC alleles (**Figure 4**). The first modification (P28Mod1) was predicted to disrupt recognition by most HLA types and murine MHC alleles, and the second, more aggressive modification (P28Mod2) was predicted to fully disrupt recognition by all HLA types and murine MHC alleles. Amino acid modifications selected for each variant have been previously made and shown to have no effects on CD21 binding, effectively decoupling the impact of mutagenesis on epitope:MHC interactions from epitope:CD21 binding.[18] Our group recently published work on the implementation of such methods to deimmunize Factor VIII (FVIII), in which we predicted and observed changes in immune responses to the modified FVIII epitopes for humanized mice and for FVIII-deficient mice.[21]

In HLA binding assays, deimmunizing modifications to the peptide representing the specific region where the P28 peptide segment overlaps with $C3d_{223-237}$ reduced peptide binding to the DRB1*0101 and 0401 alleles in comparison with the corresponding wild type sequence (**Table 3**). These results suggest that the peptide mutations may also disrupt binding to mouse MHC and reduce antigenicity of P28.

A recombinant expression construct corresponding to the HIV-1 ENV_{gp120} glycoprotein antigen fused to a 4X repeat of P28 or "deimmunized" P28Mod1 or P28Mod2 was created for *in vivo* expression and analysis of adjuvant activity.

Six- to eight-week-old BALB/c mice received 5 µg of DNA by intramuscular gene gun delivery encoding ENV_{gp120}-4XP28, ENV_{gp120}-4XP28Mod1, and ENV_{gp120}-4XP28Mod2 on weeks 0, 3, and 6. At week 8, blood was collected, and ENV_{gp120} titers were measured by enzyme-linked immunosorbent assay (ELISA). **Figure 5** shows that while immunization with ENV_{gp120} glycoprotein recombinantly expressed with wild-type P28 contributed to robust immune responses as measured by anti-ENV_{gp120} antibody titers, immune responses to ENV_{gp120} were significantly diminished when recombinantly expressed with either of the P28 deimmunized modifications (P28Mod1 and P28Mod2). In fact, a further-diminished immune response was observed with P28Mod2, the more aggressive mutation predicted to more effectively disrupt recognition by both $I\text{-}A^d$ and $I\text{-}E^d$. The results of this experiment suggest that the adjuvant activity of P28 may involve MHC binding and T cell epitope content, providing *in vivo* evidence for T cell-mediated immunogenicity in the adjuvant activity of C3d.

DISCUSSION

Adjuvants consisting of a polysaccharide antigen covalently linked to either a foreign carrier protein or to complement component C3d often enhance the generation of a primary humoral response.[1,2,3] The prevailing model suggests the adjuvant effect of C3d is due to B cell activation upon cross-linking of the antigen recognition molecules (Ig) and complement-binding molecules (CD21), resulting in a lowered activation threshold and more robust immune response.[4,15] Yet mice lacking complement receptors CD35 (CR1) and CD21 (CR2) were observed to respond to protein antigen-C3d conjugates in a similar or enhanced manner, respectively, as compared to wild-type mice[6,7] These findings challenge the current models and suggest an auxiliary immune activation pathway. We thus propose a second component to the adjuvant effect of C3d: namely, that C3d is rich in T cell epitopes and that when, in the course of antigen internalization and processing, they are presented by MHC II to autoreactive T helper cells, the T helper cells enhance antibody responses. In earlier work, we showed that C3d contains an unusually high concentration of T cell epitopes compared to other serum proteins, suggesting that T cell activation by the MHC display of C3d epitopes may be an alternative mechanism for adjuvant activity of C3d.

In the present study, we provide evidence for the role of T cell epitope content and autoreactive T cells in the development of immune responses to C3d and associated antigens. Focusing on three peptides identified in our previous work,[9] we establish that C3d epitope-specific responses (as judged by IFNγ ELISpot) are apparent in multiple donors and furthermore, donors respond to peptides in a manner consistent with HLA type as predicted by *in silico* methods and *in vitro* HLA binding assays (**Table 2**, **Figure 2**). The prior studies were conducted with non-HLA typed donors and HLA binding was performed with fewer alleles, preventing the correlations of predicted and observed T cell responses that are now established in this work.

To further test the relative contribution of T cell epitopes to the adjuvant effect of C3d, we focused on the P28 peptide segment of C3d. P28 is an attractive candidate for deimmunization since it has a single T cell epitope (overlapping with the $C3d_{223-246}$ epitope cluster identified in our studies) and has demonstrated adjuvant properties in the context of protein immunogens[20] and DNA vaccine immunogens.[5] By mutating this single T cell epitope, we effectively demonstrate the importance of T cell epitope content to the adjuvant activity of C3d. Our finding that targeted mutations to P28, predicted to disrupt MHC recognition (**Figure 4**) and significantly

decreasing the adjuvant activity of P28 *in vivo* (**Figure 5**), supports the role that T cell epitope content may have in C3d adjuvant activity.

Regions of C3d encompassing predicted T cell epitopes appear to be highly conserved throughout evolution of multicellular phyla - further highlighting their critical biological function (Matthew Ardito, personal communication). A better understanding of the biological role of these epitopes as undertaken in this study and careful evolutionary mapping may place C3d at a critical evolutionary junction where humoral immunity meets cellular responses. In fact, components of complement have been shown to be expressed by dendritic cells (critical for cellular immunity) and have further been shown to be necessary for T cell activation.[22,23] We believe this activation to be in part due to MHC presentation of C3d epitopes.

Focusing on the T cells responding to these epitopes, immunophenotyping of the C3d epitope-activated T cells points to a prominent role for CD4+CD45RO+ memory T cells circulating in the periphery in this response (**Figure 3**). Thus it appears that C3d-specific autoreactive T cells are circulating in the periphery, poised to respond to C3d associated with foreign antigens. While there are numerous examples of unwanted responses by autoreactive T cells in such auto-immune diseases as type 1 diabetes,[24] rheumatoid arthritis,[25] and multiple sclerosis,[26] these data suggest a natural, *beneficial* role for autoreactive T cells. A link between activation of complement and antibody-associated autoimmune disorders such as uveoretinitis and Systemic Lupus Erythematosis has been described.[27,28] Additionally, it is known that thymic deletion of autoreactive T cells is imperfect; 25-40% of autoreactive T cells escape into the periphery because of low TCR avidity,[29,30] absence of antigen expression in the thymus, inefficient expression, or inefficient processing.[31] This escape of autoreactive T cells to the periphery may indicate a need for them in natural immunity.

We have used our immunoinformatics tools to identify other T cell epitopes in autologous proteins that suppress immune response. Using EpiMatrix and ClustiMer, we discovered regulatory T cell epitopes (Tregitopes) that reside within IgG and likely within other autologous, high-abundance serum proteins, pointing to a very different role for Tregitope-autoreactive T cells -- in this instance, induction of natural regulatory T cells and tolerance to associated antigenic epitopes.[32] Tregitopes may serve to dampen immune response to co-administered immunogens (such as antibody idiotypes). In contrast to Tregitopes, which may serve as a universal “off switch,” our data would indicate that C3d induces an effector response to associated epitopes, acting as a universal “on switch.”

A better understanding of the C3d-specific T cell responses observed here may lead to an enhanced knowledge of the role of complement in autoimmune disorders, as well as the role of autoreactive T cells in autoimmune disease and natural defense mechanisms. Our findings are consistent with the existence of a CD21-independent pathway by which peripheral T helper cells are activated by self peptide-MHC II complexes, aiding in the adjuvant effect of C3d opsonization, and perhaps representing a critical link between humoral and cellular responses to foreign antigens.

METHODS

Immunoinformatics

T cell epitopes in human C3d were identified using the epitope-mapping algorithm EpiMatrix.[33] Updated cluster scores are referenced in this work. The 302-amino-acid sequence was parsed into overlapping 9-mer frames, each of which was analyzed against a panel of eight common HLA class II alleles that cover >90% of human populations (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, and DRB1*1501).[34] From this EpiMatrix analysis, 11 "clusters," or regions of high putative T cell epitope content, were identified, of which three candidates (17-23 amino acids in length) were chosen for synthesis. In-depth discussion of the methods used for making predictions with EpiMatrix can be found in De Groot *et. al.*[35] Frames are given Z-scores, which range from approximately -3 to +3 and are normally distributed. These scores indicate the potential of a 9-mer frame to bind to a given HLA allele. Frames containing four or more alleles scoring above 1.64 are referred to as EpiBars, and have an increased likelihood of binding to HLA. Significant EpiMatrix scores across multiple frames are then aggregated to create an EpiMatrix Cluster Immunogenicity score. Epitope clusters identified in reference 9 using EpiMatrix version 1.1 were re-analyzed in the updated version 1.2. Updated cluster scores are referenced for this work in **Table 1**.

Peptide synthesis

Peptide synthesis was conducted (21st Century Biochemicals; Marlboro, MA, USA) using solid-phase peptide synthesis employing N-(9-fluoronylmethoxycarbonyl) (Fmoc)-based chemistry and Fmoc-L-amino acid building blocks on a Rainin Symphony automated peptide synthesizer (Protein Technologies, Tucson, AZ, USA) to >80% purity. Peptides were synthesized with the amino and carboxyl ends blocked to reduce peptide degradation during incubations. Peptide sequence and purity was confirmed by MALDI/TOF MS on a QSTAR XL Pro mass spectrometer (Applied Biosystems, Grand Island, NY, USA). The peptides selected for this study (located at

amino acids 223-246, 248-265, and 269-286 of the C3d sequence) were based on EpiMatrix analyses described above (see **Table 1**).

HLA binding assays

Each of the three C3d peptides was tested in HLA binding assays to six alleles: DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101, and DRB1*1501. Class II HLA binding assays were adapted for high throughput by EpiVax from methods previously described by Kwok and colleagues.[36] Non-biotinylated test peptide over a range of concentrations (2-128 μM) competed against a biotinylated tracer peptide (0.1-1 μM) for binding to purified class II HLA molecules (0.5-2 μg/ml) in 96-well plates at 37°C for 24 hours. Pan anti-class II antibodies (L243, anti-HLA-DR) were used to capture class II complexes on ELISA plates, which were developed using streptavidin-europium and read on a time-resolved fluorescence plate reader. Nonlinear regression analysis was performed, and half maximal inhibitory concentrations (IC50) values were calculated.

Blood samples

Interferon gamma ELISpot assays were performed using cryopreserved peripheral blood mononuclear cells (PBMCs) separated by Ficoll density gradient centrifugation of whole blood. Healthy human subjects were recruited from Clinical Partners in Johnston, RI, USA in accordance with all federal guidelines and institutional policies. Institutional review boards in Providence, RI approved the informed consent procedures and research protocols**.** Informed consent was obtained prior to obtaining all samples for this study.

ELISpot assays

Cryopreserved PBMCs from four healthy human donors with HLA types DRB1*0301, DRB1*0401, DRB1*0701, and DRB1*1101 were used to determine the frequency of epitope-specific T lymphocytes. Purified PBMCs were stimulated on day 1 with either pan HLA DR-binding epitope (PADRE) or a pool of three C3d-derived peptides at a final total peptide concentration of 30 μg/ml in RPMI 1640+GlutaMax (Life Technologies; Grand Island, NY, USA) supplemented with 10% human AB serum (Valley Biomedical; Winchester, VA, USA), 1% L-glutamine (Life Technologies), 1% 1M HEPES (Life Technologies), and 50 μg/ml Gentamycin (Sigma-Aldrich; St. Louis, MO, USA). Cells were plated at 5 million cells per well in a 12-well plate and incubated for 10 days at 37°C and 5% CO_2. IL-2 (10 ng/ml) and IL-7 (20 U/ml), both manufactured by BioSource (Nivelles, Belgium), were added to each of the wells on days 1 and

5 of the incubation period, and a half-medium exchange was performed on day 5. On day 10, PBMCs were harvested and added at 2.5 x 10^5 cells per well to a Mabtech® IFNγ ELISpot plate pre-coated with anti-IFNγ antibody (Mabtech; Nacka, Sweden). Cells were re-stimulated in triplicate in the ELISpot plate with 10 μg/ml of one of the three individual C3d peptides, 30 μg/ml of PADRE, 30 μg/ml of the C3d peptide pool, or 2 μg/ml PHA (positive control). Twelve wells of cells without re-stimulation were used to measure assay background. Plates were incubated at 37°C and 5% CO_2 for 48 hours and were developed according to the manufacturer's instructions. To develop, plates were washed and biotinylated anti-IFNγ was added; after a two-hour incubation, plates were washed again, and streptavidin-HRP was added. Plates were incubated for one hour, washed, and developed by addition of tetramethylbenzidine (TMB) substrate. The frequency of antigen-specific cells was calculated as the number of spots per 10^6 cells seeded. Responses were considered positive if the number of spots was significantly greater by Student's t-test than 50 spot forming cells per well (1 spot over background per 20,000 PBMCs).

Immunophenotyping

PBMCs from a single DRB1*0701-positive donor (Clinical Partners; Johnston, RI, USA) were stimulated with a C3d peptide pool (comprised of $C3d_{223-246}$, $C3d_{248-265}$, and $C3d_{269-286}$) at 30 μg/ml or with MOG_{35-55} peptide at 30 μg/ml for 10 days under the same conditions as the ELISpot preculture. On day 10, PBMCs were stimulated with the C3d peptide pool, MOG_{35-55}, CytoStim (Miltenyi Biotec; Bergisch Gladbach, Germany), or media alone for three hours. After secondary stimulation, IFNγ-positive cells were enriched using an IFNγ secretion assay kit (Miltenyi) and then co-stained with anti-human antibodies for CD4-FITC, CD45RA-PerCP, and CD45RO-APC, according to the manufacturer's recommendations (eBioscience; San Diego, CA, USA). Samples were acquired on a Beckton Dickinson (Franklin Lakes, NJ, USA) FACSCalibur flow cytometer. In FlowJo analysis software (FlowJo, Ashland, OR, USA), CD4+, IFNγ+ cells were identified from the live lymphocytes, and proportions of CD45RA+ and CD45RO+ cells were examined within this double-positive population.

Mouse studies

BALB/c mice (*Mus musculus*, female, 6–8 weeks) were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA), housed in microisolator units with free access to food and water, and cared for under United States Department of Agriculture guidelines for laboratory animals. Mice (n=5 per group) were gene gun-immunized on shaved abdominal skin using the hand-held Bio-

Rad gene delivery system. Mice were immunized with two gene gun doses (at weeks 0, 3 and 6) containing 5 μg of DNA (encoding ENV_{gp120}-4xP28, ENV_{gp120}-4xP28Mod1, or ENV_{gp120}-4xP28Mod2) per 0.5 mg of approximately 1 μm gold beads (Bio-Rad; Hercules, CA, USA) at a helium pressure setting of 400 lb/in^2. Fourteen days after the third vaccination (week 8), blood was collected from anesthetized mice via the retro-orbital plexus and transferred to a microfuge tube. For blood sample collection, animals were anesthetized with a mixture of ketamine/xylazine. Tubes were centrifuged, and sera was removed and frozen at -20 ± 5°C. ENV_{gp120} antibody titers were measured by ELISA. P28Mod1 alone was used as a negative control, and codon optimized gp120 (cogp120), which expresses 7-10 times more gp120 protein than wtgp120 at the same DNA dose[37], was used as a positive control. For data processing, the single highest responding mouse in every group was removed from analysis. All procedures were in accordance with the NRC Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

ELISA

The ELISA was used to assess total antibody titer to the HIV-1 envelope. High-binding, 96-well polystyrene plates (Costar; Lowell, MA, USA) were coated overnight with 50 ng/well of recombinant ENV. Plates were blocked with 5% milk diluted in PBS with 0.05% Tween 20. Serum samples were diluted in blocking buffer and added to plates. Serum was two-fold serially diluted and allowed to incubate for 1 hour at room temperature. Plates were washed, and horseradish peroxidase (HRP)-linked species-specific antibody against IgG was diluted in blocking buffer and added to plates. Plates were incubated for 1 hour at room temperature. Plates were washed, and HRP was developed with TMB substrate (Sigma-Aldrich). Plates were incubated in the dark for 15 minutes, and then the reaction was stopped with 2N H_2SO_4. Optical densities at a wavelength of 450nm (OD_{450}) were read by a spectrophotometer (BioTek; Winooski, VT, USA), and endpoint dilution titers were determined as the reciprocal dilution of the last well which had an OD_{450} above the mean OD_{450} plus two standard deviations of naïve animal sera.

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CONFLICT OF INTEREST

Eight of the coauthors on this manuscript are employees of EpiVax (ADG, LL, TMJ, RT, CB, AV, LM, WM), and two (ASDG, WM) are majority stockholders. These authors recognize the presence of a potential conflict of interest and affirm that the descriptions of experiments represented in this paper are original and unbiased observations.

FIGURES AND TABLES

P28 ($C3d_{208-235}$): KFLTTAKDKNRWEDP**G*K*Q*LY*NVEATSYA**

$C3d_{223-246}$: **GKQLYNVEATSYA**LLALLQLKDFD

Figure 1. The T cell epitope region of C3d residues 223-246 overlaps with P28. The T cell epitope shared between P28 and $C3d_{223-246}$ is shown in bold. Amino acids in italics and underlined were mutated. Solid underline denotes amino acids mutated to alanine in P28Mod1. The additional K>N mutation in P28Mod2 is denoted by the dotted underline. The P28 region, which contains the major binding site of C3d to CD21,[13] shares 13 amino acids with C3d.

Fig.2a

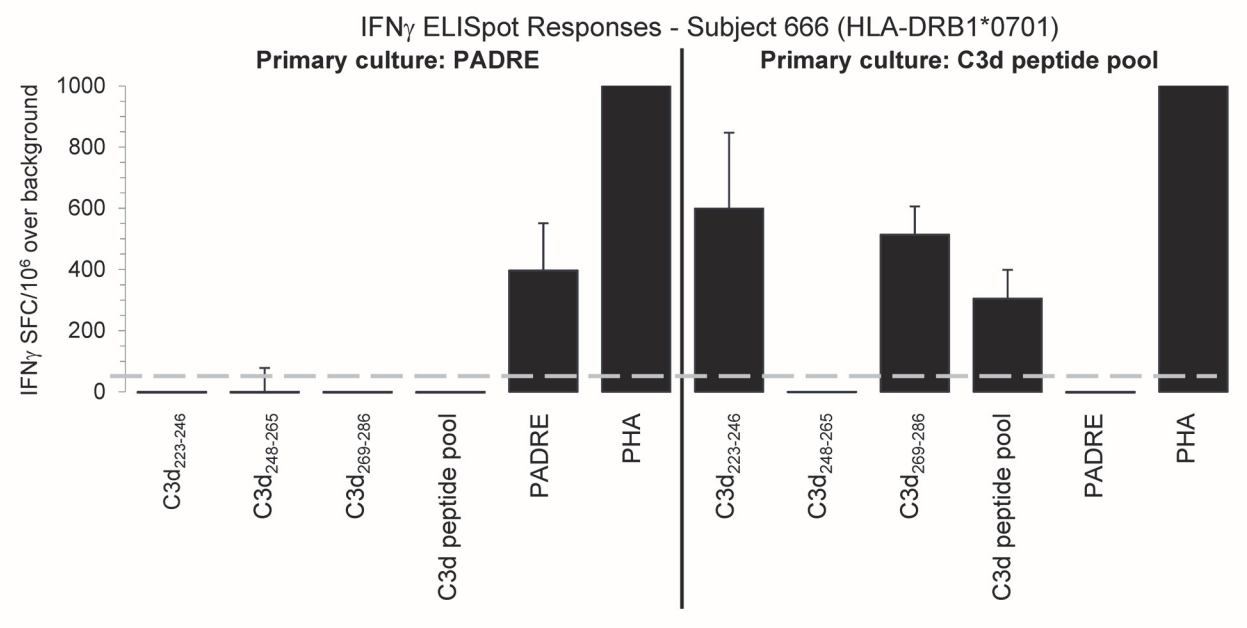
IFNγ ELISpot Responses - Subject 666 (HLA-DRB1*0701)
Primary culture: PADRE
Primary culture: C3d peptide pool
IFNγ SFC/10^6 over background
1000
800
600
400
200
0
C3d223-246
C3d248-265
C3d269-286
C3d peptide pool
PADRE
PHA

Fig.2b

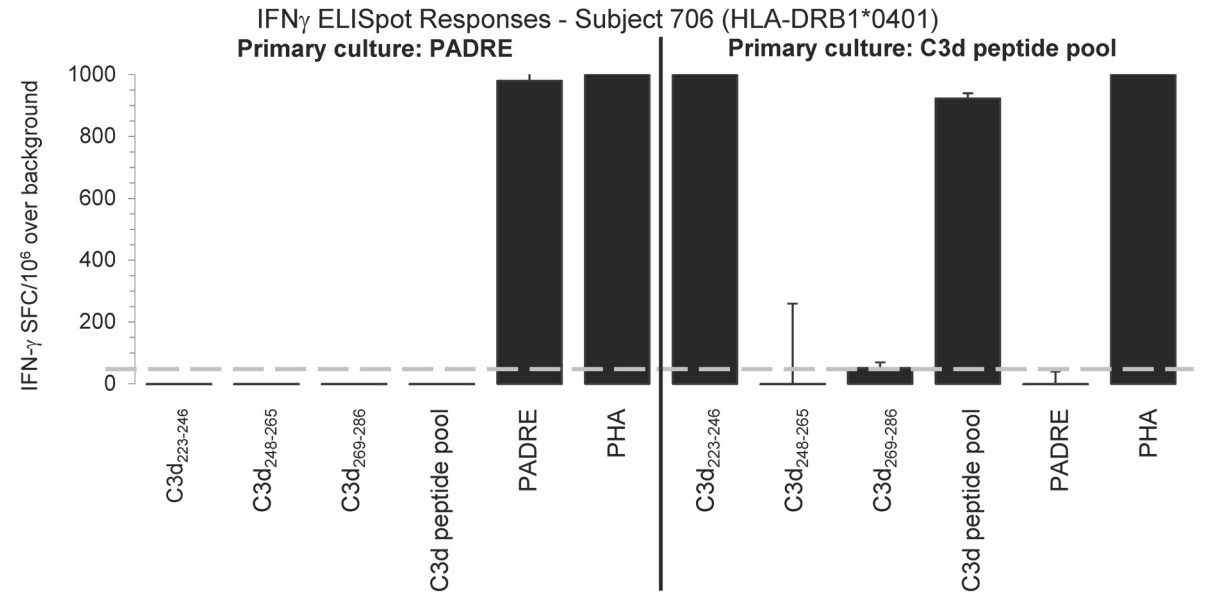
IFNγ ELISpot Responses - Subject 706 (HLA-DRB1*0401)
Primary culture: PADRE
Primary culture: C3d peptide pool
IFN-γ SFC/10^6 over background
1000
800
600
400
200
0
C3d223-246
C3d248-265
C3d269-286
C3d peptide pool
PADRE
PHA

Fig.2c

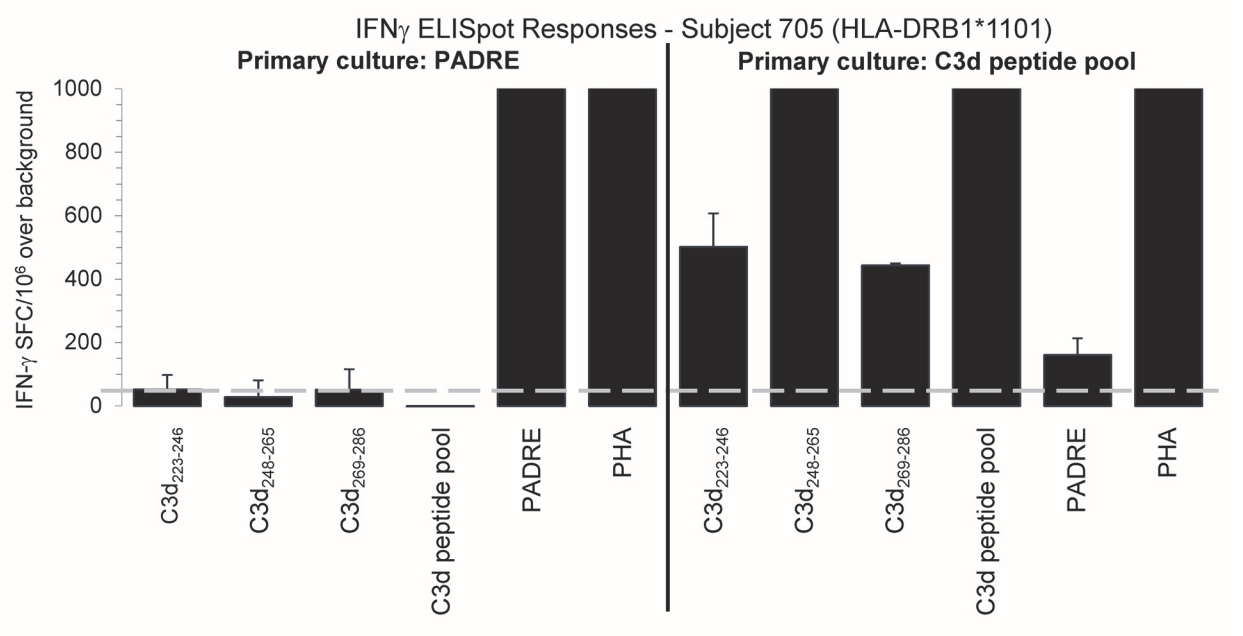
IFNγ ELISpot Responses - Subject 705 (HLA-DRB1*1101)
Primary culture: PADRE
Primary culture: C3d peptide pool
IFN-γ SFC/10^6 over background
1000
800
600
400
200
0
C3d223-246
C3d248-265
C3d269-286
C3d peptide pool
PADRE
PHA

Fig.2d

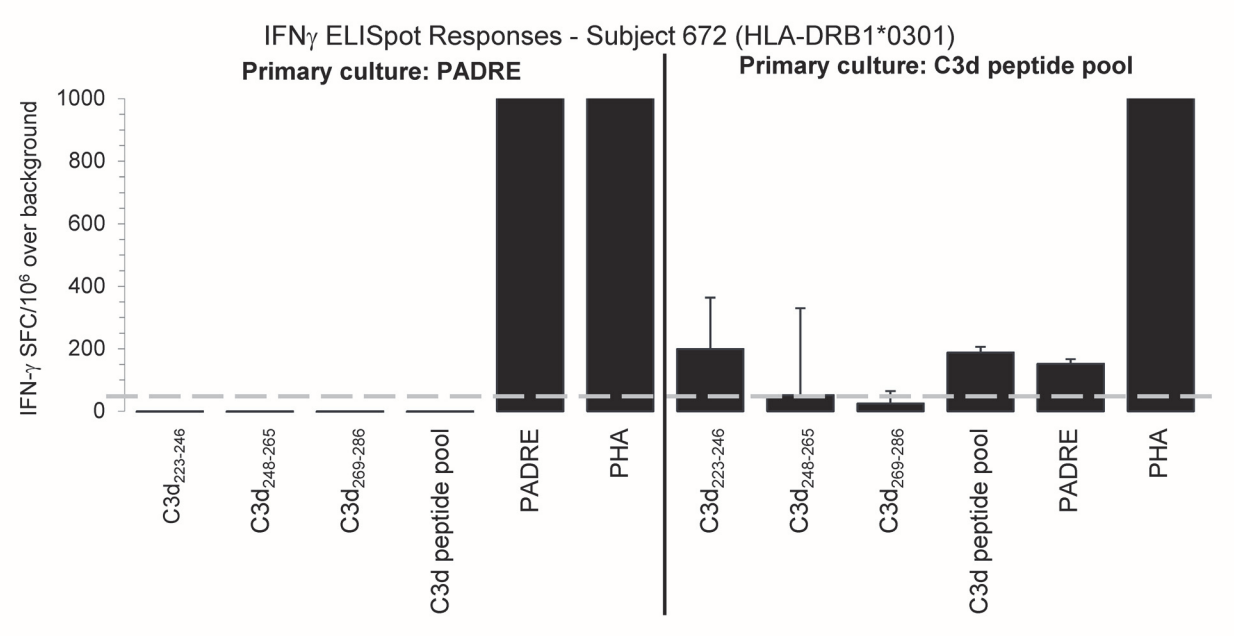
IFNγ ELISpot Responses - Subject 672 (HLA-DRB1*0301)
Primary culture: PADRE
Primary culture: C3d peptide pool
IFN-γ SFC/10^6 over background
1000
800
600
400
200
0
C3d223-246
C3d248-265
C3d269-286
C3d peptide pool
PADRE
PHA

Figure 2. PBMCs from healthy donors respond specifically to C3d epitope clusters. Frozen PBMC samples from four healthy donors were thawed and stimulated for 10 days with either a pool of human C3d epitope peptides, a non-specific control peptide (PADRE), or a mitogen positive control (PHA). Cells were re-stimulated in a 48-hour IFNγ ELISpot with either individual peptides or pools. A response was considered positive if the number of spots was greater than or equal to 50 spot forming cells over background per 10^6 PBMCs (grey dotted line). HLA type of donors is shown at the top of each graph. PBMCs from all donors responded to C3d peptides when cultured with the C3d peptide pool, but not with the PADRE non-specific control peptide.

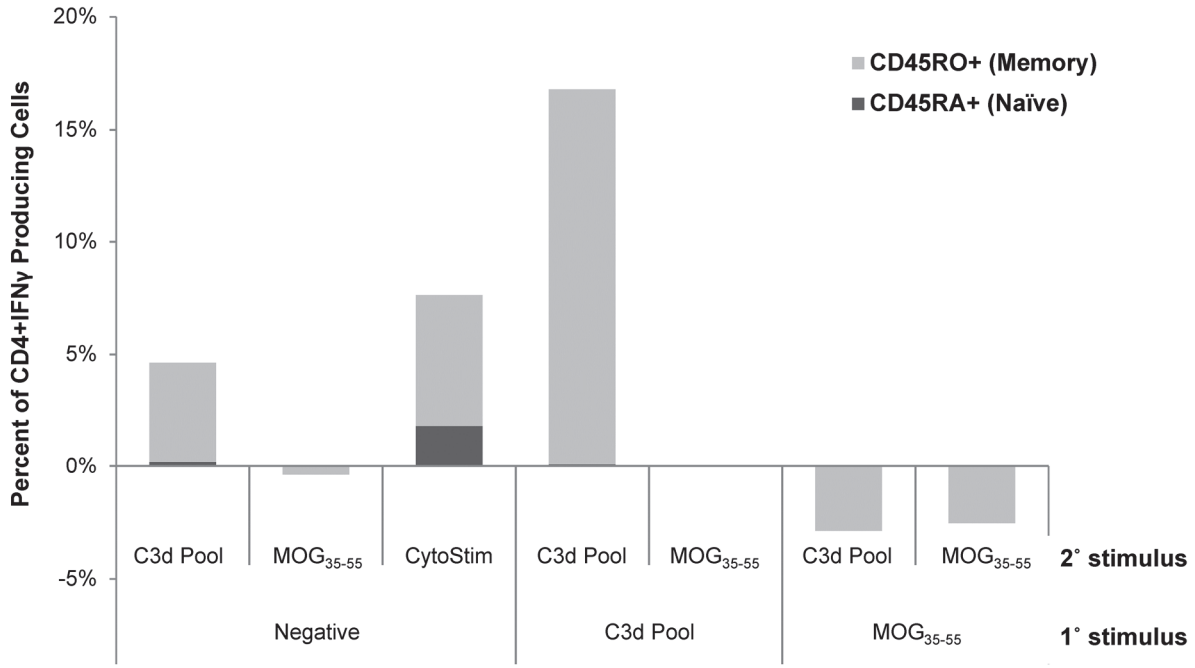

Figure 3. C3d epitopes specifically induce a memory T cell response. Primary stimulations were performed as in Figure 2. Secondary stimulations were performed with media, C3d peptide pool, MOG_{35-55} or CytoStim. IFNγ-secreting cells were then captured, magnetically separated and labeled with antibodies for CD4, CD45RA (naïve marker) and CD45RO (memory marker). Cells were first gated for CD4+IFNγ+, then CD45RA and CD45RO. Each primary stimulation group above is subtracted for background with secondary stimulation of media alone. Incubation and re-stimulation with the C3d peptide pool produced a significant increase in the CD4+IFNγ+CD45RO+ memory T cell subpopulation, as well as for the C3d pool secondary stimulus of unstimulated cells, but not for the MOG negative control peptide restimulation under any primary stimulus condition.

P28

Frame	AA Sequence	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	I-A^b Z-Score	I-A^d Z-Score	I-E^d Z-Score	Hit Count
1	KFLTTAKDK	0.35	-1.12	0.25	0.64	0.30	-0.27	-0.07	-0.05	-0.79	1.01	0.59	0
2	FLTTAKDKN	0.85	0.11	0.11	0.02	1.13	0.71	0.40	0.58	1.08	-1.35	0.29	0
3	LTTAKDKNR	0.17	0.93	0.75	0.53	0.93	0.08	1.11	1.08	1.07	0.10	0.85	0
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	KQLYNVEAT	0.14	-0.77	0.46	0.85	0.81	0.23	0.10	0.06	-0.11	1.18	0.27	0
18	QLYNVEATS	0.04	0.10	0.42	-0.58	0.55	1.13	0.32	-0.67	0.43	-0.16	-0.44	0
19	LYNVEATSY	1.23	1.65	0.83	0.67	-0.05	0.15	1.82	1.14	1.35	1.25	1.88	3
20	YNVEATSYA	1.75	1.24	2.37	1.26	0.30	1.30	0.90	0.23	2.40	0.52	-0.62	3

P28Mod1

Frame	AA Sequence	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	I-A^b Z-Score	I-A^d Z-Score	I-E^d Z-Score	Hit Count
1	KFLTTAKDK	0.35	-1.12	0.25	0.64	0.30	-0.27	-0.07	-0.05	-0.79	1.01	0.59	0
2	FLTTAKDKN	0.85	0.11	0.11	0.02	1.13	0.71	0.40	0.58	1.08	-1.35	0.29	0
3	LTTAKDKNR	0.17	0.93	0.75	0.53	0.93	0.08	1.11	1.08	1.07	0.10	0.85	0
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	KQ**AA**NVEAT	0.90	0.25	1.16	1.16	0.62	0.41	-0.23	0.06	1.18	1.98	0.27	1
18	Q**AA**NVEATS	-0.34	-0.28	0.05	-0.96	0.16	0.74	-0.05	-1.03	0.93	0.46	0.09	0
19	**AA**NVEATSY	-0.10	0.26	-0.45	-0.63	-1.44	-1.20	0.46	-0.19	1.06	1.43	1.23	0
20	**A**NVEATSYA	0.29	0.18	0.96	-0.19	-1.23	-0.20	-0.13	-0.78	1.17	0.16	-1.14	0

P28Mod2

Frame	AA Sequence	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	I-A^b Z-Score	I-A^d Z-Score	I-E^d Z-Score	Hit Count
1	KFLTTAKDK	0.35	-1.12	0.25	0.64	0.30	-0.27	-0.07	-0.05	-0.79	1.01	0.59	0
2	FLTTAKDKN	0.85	0.11	0.11	0.02	1.13	0.71	0.40	0.58	1.08	-1.35	0.29	0
3	LTTAKDKNR	0.17	0.93	0.75	0.53	0.93	0.08	1.11	1.08	1.07	0.10	0.85	0
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	N**Q****AA**NVEAT	0.71	-0.05	0.98	0.98	0.42	0.22	-0.52	-0.22	0.99	1.58	0.46	0
18	Q**AA**NVEATS	-0.34	-0.28	0.05	-0.96	0.16	0.74	-0.05	-1.03	0.93	0.46	0.09	0
19	**AA**NVEATSY	-0.10	0.26	-0.45	-0.63	-1.44	-1.20	0.46	-0.19	1.06	1.43	1.23	0
20	**A**NVEATSYA	0.29	0.18	0.96	-0.19	-1.23	-0.20	-0.13	-0.78	1.17	0.16	-1.14	0

Figure 4. Targeted mutation of the single T cell epitope in P28 abolishes predicted binding to HLA and MHC alleles. EpiMatrix cluster reports show the P28, P28Mod1 and P28Mod2 sequences parsed into overlapping 9-mer frames. Each 9-mer is analyzed for predicted recognition by eight common HLA haplotypes and MHC alleles I-A^b, I-A^d and I-E^d. Z-Scores above 1.64 are predicted to bind respective alleles. Epitope/HLA matches are shown in shaded boxes. Modified amino acids in P28Mod1 and P28Mod2 are underlined. The P28Mod1 variant is predicted to disrupt binding to most MHC alleles, and the more aggressive P28Mod2 variant is predicted to fully disrupt binding.

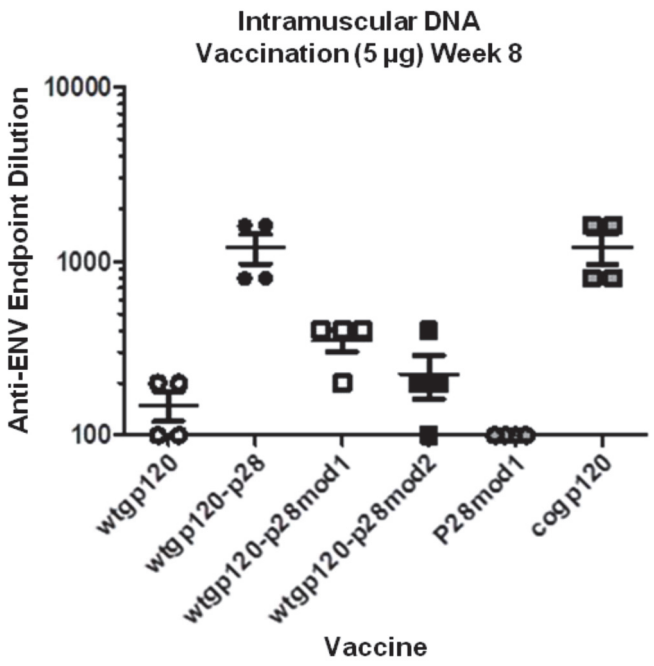

Figure 5. Mutation of the single T cell epitope in P28 significantly diminishes its adjuvant activity. The immunogenicity of wild-type gp120 (wtgp120; open circles) is enhanced when conjugated to a 4X concatamer of P28 (wtgp120-p28; black circles) as measured by anti-Env titers. When conjugated to a 4X concatamer of P28 mutated at the single predicted T cell epitope (wtgp120-p28mod1, wtgp120-p28mod2; open squares and black squares, respectively) the adjuvant activity of P28 is significantly diminished. P28mod1 alone was used as a negative control. Codon optimized gp120 (cogp120), which expresses 7-10 times more gp120 protein than wtgp120 at the same DNA dose[37], was used as a positive control. Immunization with ENVgp120 glycoprotein conjugated to p28mod1 or p28mod2 significantly diminished antibody responses in comparison with the wild-type P28 conjugate.

Peptide name	Start and stop in mature C3d	Equivalent start and stop in C3	Sequence	EpiMatrix cluster score
$C3d_{223-246}$	223-246	1224-1247	GKQLYNVEATSYALLALLQLKDFD	7.12
$C3d_{248-265}$	248-265	1249-1266	VPPVVRWLNEQRYYGGGY	6.24
$C3d_{269-286}$	269-286	1270-1287	QATFMVFQALAQYQKDAP	23.01

Table 1. C3d peptide locations, sequences, and cluster scores. Amino acids were numbered according to their position within C3d (N terminus to C terminus); position 1 of C3d corresponds to position 1002 of C3. EpiMatrix cluster scores are a measure of epitope promiscuity (probability to be recognized by multiple HLA alleles). Potentially immunogenic epitopes tend to have higher EpiMatrix cluster scores.

Peptide name	HLA-DRB1 Allele	EpiMatrix Z-score	Prediction	Binding IC^{50} (μM)
$C3d_{223-246}$	0101	1.75	binder	<2
	0301	1.65	binder	13.96
	0401	2.37	binder	<2
	0701	1.62	non-binder	(<2)
	1101	1.96	binder	<2
	1501	1.92	binder	85.07
$C3d_{248-265}$	0101	1.90	binder	(non-binder)
	0301	1.80	binder	(non-binder)
	0401	1.63	non-binder	non-binder
	0701	0.72	non-binder	non-binder
	1101	2.28	binder	<2
	1501	1.35	non-binder	non-binder
$C3d_{269-286}$	0101	1.69	binder	5.63
	0301	1.72	binder	77.63
	0401	2.68	binder	9.64
	0701	1.70	binder	2.45
	1101	2.37	binder	<2
	1501	2.43	binder	>100

Table 2. EpiMatrix predictions correlate well with *in vitro* HLA binding. Peptides were predicted to bind a given HLA allele if their EpiMatrix Z-score was >1.64. Binding results that differ from predicted results are in parenthesis. Binding results confirmed EpiMatrix predictions for 83% of the peptide/HLA allele combinations.

Peptide ID	Peptide Sequence, $C3d_{223-237}$	Binding IC_{50} Values (nM) by HLA Allele		
		DRB1*01:01	DRB1*03:01	DRB1*04:01
WT	GKQLYNVEATSYALL	365	2396	170
MOD1	GKQ**AA**NVEATSYALL	582	2873	5202
MOD2	G**N**Q**AA**NVEATSYALL	992	1208	4407

Table 3. HLA binding of the single T cell epitope in P28 and its deimmunized variants. HLA binding was assessed for the sequence overlapping the P28 peptide segment and $C3d_{223-237}$ (Figure 1) and two variants designed to disrupt binding. Assays were performed for HLA alleles predicted to bind the wild type sequence. Modified amino acids in peptide sequences are underlined and in bold. Binding affinities are expressed as IC_{50} values in nM. The variant (MOD1 and MOD2) mutations reduced peptide binding to HLA DRB*0101 and *0401, as indicated by higher IC_{50} values.

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