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Generation of a universal CD4 memory T cell recall peptide effective in humans, mice and non-human primates

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ABSTRACT

CD4T cells play a key role in humoral immunity by providing help to B cells, enabling effective antibody class switching and affinity maturation. Some vaccines may generate a poor response due to a lack of effective MHC class II epitopes, resulting in ineffective helper T cell activation and recall and consequently poor humoral immunity. It may be beneficial to provide a CD4 T cell helper peptide with a vaccine particularly in the case of a poorly immunogenic antigen. Such a T cell helper peptide must be promiscuous in its ability to bind a broad range of MHC class II alleles due to broad allelic variation in the human population. We designed a chimeric MHC class II peptide (TpD) with epitopes from tetanus toxoid and diphtheria toxoid, separated by an internal cathepsin cleavage site. TpD was capable of inducing a memory recall response in peripheral blood mononuclear cells from 20/20 human donors. T cells responding to TpD showed a central memory phenotype. Immunization of mice with a synthetic nicotine nanoparticle vaccine containing TpD showed that the peptide was required for robust antibody production and resulted in a long term CD4 memory T cell recall response.

As a pre-clinical model two non-human primate species, rhesus macaques and cynomolgus monkeys, were immunized with a nicotine nanoparticle vaccine and evaluated for an anti-nicotine antibody response and TpD specific memory T cells. We found that 4/4 rhesus monkeys had both sustained antibody production and TpD memory T cells for the duration of the experiment (119 days). In addition 30/30 cynomolgus monkeys dosed with nicotine vaccine nanoparticles showed dose-dependent antibody generation and T cell recall response compared to saline injected controls. In summary we have developed a potent universal memory T cell helper peptide (TpD) that is active in vitro in human PBMCs and in vivo in mice and non-human primates.

1. Introduction

CD4 T cells provide 'help' in stimulating B cells to mature as well as undergo immunoglobulin class switching and affinity maturation, and as a result are required for development of a successful vaccine.

In order to provide help CD4 T cells must recognize HLA Class II epitopes found in the immunogen. Unfortunately not all vaccines have sufficient HLA Class II epitopes to induce a proper T cell helper response in a diverse population. As a consequence there may be some value in designing a 'universal' helper T cell epitope to be included in the vaccine. A limiting factor for targeting a specific CD4 response to induce T cell help in a vaccine is the large number of polymorphisms in MHC class II genes. Each individual has specific set of MHC class II alleles, and each allele may have different peptide-binding properties [1]. As a consequence, a universal CD4 T cell helper peptide would have to bind promiscuously to multiple alleles to provide broad coverage across a population. In addition, the peptide would preferably make use of pre-existing CD4 T cell memory to give a rapid and robust response. The concept of the need for a 'promiscuous' or universal helper peptide has been studied by a number of groups. Efforts have primarily focused on de novo designed peptides [2] or identifying peptides that provide a recall response to epitopes in commonly administered vaccines, such as tetanus and diphtheria toxoid (TT and DT respectively), in previously vaccinated subjects. The latter approach has the advantage of being able to validate peptide selection by assessing in vitro recall responses using peripheral blood mononuclear cells (PBMC) from normal healthy donors. While a broad range of potential universal epitopes have been identified for both TT and DT [3–7],

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a considerable amount of work has focused on $TT_{830-844}$ [8–10]. Experimental evidence suggests that $TT_{830-844}$ can be presented by up to ten different MHC class II alleles [3,6], although this has been disputed [11]. $TT_{830-844}$ has been used as a helper peptide in various animal species including mice [12–14], rats [15], rabbits [16] and rhesus macaques [17]. The predominant focus has been on using a helper peptide to improve a cytotoxic T lymphocyte (CTL) response for treating viruses and cancer [13–15], rather than for enhancement of humoral immunity. In one primate study, a CTL response was induced to simian immunodeficiency virus peptides from Nef and Gag proteins [17]. However, only two of eight primates demonstrated a proliferative response to the peptide. Helper peptides have been used in several clinical studies, again primarily focusing on inducing CTL responses for the treatment of human viruses or cancer. $TT_{830-844}$ has been tested in vaccines to induce CTL responses for treatment of chronic hepatitis B virus [18] and human immunodeficiency virus infection [19–21]. In addition, the helper peptide has been used to enhance CTL responses for the treatment of melanoma [22,23]. Those publications demonstrating recall response to $TT_{830-844}$ report an average range of 60–75% of subjects responding [18,22]. However, in one report 91% of patients that received an immunization containing MHC class I restricted melanoma peptides plus $TT_{830-844}$ demonstrated a recall response to the helper peptide, but 18% that did not receive the helper peptide also responded, presumably due to previous immunization with TT [23].

We have rationally designed a fully synthetic nanoparticle-based vaccine against nicotine for smoking cessation. However neither the B cell antigen, nicotine nor the nanoparticle polymer contain T cell epitopes needed to provide help for B cell differentiation and antibody affinity maturation. Here we describe a 'universal' memory CD4 helper peptide that was designed and included in synthetic nanoparticle vaccines to provide promiscuous binding to a broad range of the most common MHC class II alleles in order to provide CD4 T cell help for B cell maturation and antibody production. We hypothesized that inclusion of a dimeric CD4 helper memory peptide (TpD) containing both TT and DT epitopes linked by a cathepsin linkage site, would result in improved antibody responses. We demonstrate that all 20 of tested normal human blood donors generated an in vitro memory recall response to the chimeric peptide. In mice the helper peptide was required to for efficient antibody response to a synthetic nicotine vaccine. In cynomolgus and rhesus monkeys high levels of antibodies could be achieved in a dose dependent fashion, with a robust memory CD4 recall response to TpD in all animals that received sufficient doses of vaccine.

2. Materials and methods

2.1. Mouse experiments

For mouse experiments female 6–8-week-old Balb/C mice (Jackson Laboratories) were housed and handled at Vivisource (Cambridge, MA) in accordance to Institutional Animal Care and Use Committee (IACUC) requirements. For vaccine injections, mice were injected subcutaneously with a single bolus of nanoparticle preparations in PBS (50 μl/limb). Mice were injected 3 times (1 prime and 2 boosts immunizations) with 2-week intervals between immunizations. For serum collection, blood was collected by lateral tail vain bleeding 12 days after each immunization and after that as indicated. At the termination of the experiment, mice were euthanized by CO_2 asphyxiation and blood collected by cardiac puncture.

For long term memory recall assays Balb/C mice were inoculated on days 0, 14 and 28 with nicotine nanoparticles containing R848 and either TpD or ovalbumin 323–339 (Ova) peptide. Spleens were harvested between 122 and 152 days after final inoculation and both CD4+ and CD11c+ cells were isolated directly ex vivo by MACS cell separation system (Miltenyi, Cambridge, MA). Cells were incubated at 37 °C at a 10:1 ratio (500,000 CD4 T cells to 50,000 dendritic cells) with 10uM peptide. Supernatants were harvested 18 h later and assayed for IFN-γ by ELISA.

2.2. Non-human primate experiments

For Rhesus macaques (Macaca mulatta) experimental procedures as outlined in Harvard Medical Associates standing committee on animal's protocol # 04758 were followed throughout the study. The study followed The Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, and was administered in accordance with IACUC requirements. Four, three year old Rhesus macaques received a total of three vaccinations at 4-week intervals. At each procedure time point, the animals were sedated with 10 mg/kg ketamine-HCl administered intramuscularly. 1 mL of the test substance was administered via the subcutaneous route. Briefly, the skin on the quadriceps was shaved, wiped with alcohol and allowed to dry. The immunizing material was then administered via a 23 gauge, 1-inch needle. The animals were monitored and returned to their home cage when awake. The animals were weighed when sedated for each procedure. Blood samples (in 10 mL round bottom tubes with EDTA; used for ELISPOT) and 5 mL of serum (used for antibody analysis) were collected at approximately bi-weekly intervals.

For the cynomolgus monkey study, animal welfare was in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1–3). The Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Academy Press, Washington, D.C., 1996, was followed. The non-clinical laboratory (MPI Research, Inc. Mattawan, MI) study was conducted in accordance with the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58. MPI Research is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International), and was under guidance of IACUC. Vaccinations with the nanoparticle vaccine and saline control were administered by injection between the skin and underlying layers of tissue in the thigh region of each animal. The same injection site on each animal was used for each administration unless a reaction at the injection site indicated that another site must be used. All injection sites were marked and identified throughout the course of the study. The dose was administered by bolus injection. Monkeys were immunized ($N = 10$ per group) on days −78 and −48 with a combined pediatric diphtheria/tetanus toxoid vaccine, and then immunized on days 1, 29, and 57 with saline, or escalating doses of 1 mL of nanoparticle vaccine at 0.5, 2.0, 8.0 and 16.0 mg/mL. Blood was collected on days shown, prior to immunization (day 1) and then on days 29, 57, 85, 113, and 141 to test for anti-nicotine antibodies. Peripheral blood was collected on day 85 for T cell recall analysis (3 mL) and PBMC isolated by percoll centrifugation.

2.3. Peripheral blood mononuclear cell preparation

Briefly, human peripheral blood mononuclear cells (PBMCs) were isolated from normal human donors (Research Blood Components, Cambridge, MA). Blood was diluted 1:1 in phosphate buffered saline and then 35 mL overlaid on top of 12 mLs Ficoll-Paque premium (GE Healthcare, Pittsburgh, PA) in a 50 mL centrifuge tube. Tubes were spun at 1400 RPM for 30 min, and the transition phase PBMCs collected, diluted in PBS with 2% fetal calf serum and spun at 1200 rpm for 10 min. Cells were re-suspended

in cell freezing media (Sigma–Aldrich, St. Louis, MO) and immediately frozen at −80 °C. For long term storage, cells were transferred to liquid nitrogen. For rhesus monkey PBMC isolation the protocol was the same except 5 mL of blood was collected and processed. For cynomolgus monkey PBMC, 3 mL of blood was processed, buffy coat was collected and overlaid on 60% Percoll (GE Healthcare), centrifuged 30 min at 1755 rpm, washed and frozen as described above.

2.4. Tissue culture and flow cytometry

Frozen PBMC were thawed (37 °C water bath), re-suspended in PBS 10% FCS, spun down and re-suspended to 5×10^6 cells/mL in tissue culture media (RPMI), supplemented with 5% heat inactivated human serum (Sigma–Aldrich), L-glutamine, penicillin and streptomycin, (Gibco, Grand Island, NY). For memory T cell recall response assays, cells (0.6–1.0 mL) were cultured in 24-well plates with 4 μM peptide (GenScript) at 37 °C 5% CO_2 for 2 h. One μL of 1000× Brefeldin A (BD, San Jose, CA) per mL of culture media was then added and cells returned to a 37 °C incubator for 4–6 h. Cells were then incubated at 27 °C, 5% CO_2 for 16 h. Detection of activated memory T cells was performed by incubation of cells with fluorochrome conjugated antibodies: CD4-FITC, CD45RA-PE, CD62L-Cy7PE, CD45RO-PerCPCy5.5, CCR7-PB (Biolegend, San Diego, CA) and CD45RA-PE, CD4-APCCy7, CD27-V500 (BD) followed by membrane permeabilization and fixing (BD). Expression of intracellular cytokines was detected using interferon-γ-APC and TNF-α-APC (BD). 200,000–500,000 cells were then analyzed using a either a FACSCaliber (BD) or FACSCanto flow cytometer, and Cellquest or Diva (BD) software.

2.5. ELISPOT assay

For the ELISPOT assay 96 well filter plates (Millipore, Billrica, MA) were coated 18 h prior to use with PBS containing 15 μg/mL interferon-γ capture antibody (Mabtech, Mariemont, OH) at 4 °C. The plates were coated for 2 h at room temperature with complete culture media to block non-specific binding. PBMC were diluted to $3–5 \times 10^6$ cells/mL and 100 μL plated per well on the antibody pre-coated elispot plates with or without addition of 15 μM peptide (TpD). Positive control wells were stimulated with 10 μg/mL phytohemagglutinin (PHA (Sigma). After 18 h of incubation at 37 °C, elispot plates were washed in PBS containing 0.05% Tween 20 (Fisher Scientific, Waltham, MA), followed by incubation with 100 μL biotinylated anti-IFN-γ secondary antibody for 2 h at room temperature. Elispot plates were then washed 3 times in PBS/tween-20 buffer (Fisher Scientific) and three times in PBS. IFN-γ spots were developed using 100 μL per well 3-amino-9-ethylcarbazole (Sigma), dimethylformamide (Sigma) and hydrogen peroxide (Sigma) in acetate buffer. After 5–10 min of development, plates were thoroughly washed in water and dried. Interferon-γ positive elispot counts were scored by an outside vendor (ZellNet, Fort Lee, NJ). Statistical analysis was performed in Excel, and data plotted using SigmaPlot.

2.6. Nicotine nanoparticle synthesis

The nicotine nanoparticle is generated using a double emulsion process. A primary water-in-oil emulsion is formed by high shear mixing of a primary aqueous solution (TpD in 60% lactic acid) and an organic solution containing polylactic acid-polyethylene glycol-nicotine (PLA-PEG-nicotine), poly(lactic-co-glycolic acid)-R848, and PLA in dichloromethane at controlled speeds and temperatures. The double emulsion (water-in-oil-in-water) is formed by adding a secondary aqueous solution (phosphate buffer with 10% polyvinyl alcohol) to the primary emulsion and high shear mixing at controlled speeds and temperatures for a fixed duration. The PVA and phosphate buffer solution form the continuous phase. The nanoparticles are formed and hardened by evaporation of the organic solvent (dichloromethane) from a well-stirred suspension. As the solvent is removed from the emulsion, the polymeric matrix condenses and hardens into nanoparticles. The nanoparticles are further washed in PBS, and the final nanoparticle suspension is passed through a 0.2 μm filter.

2.7. ELISA protocol

ELISA plates were coated with 100 μL per well of a polylysine–nicotine conjugate in PBS and incubated overnight at 4 °C. Plates were washed 3 times in wash buffer (PBS/0.05%Tween20) (Sigma) and blocked with 300 μL per well of 1% casein (Fisher Scientific) in PBS for 2 h at room temperature. Plates were washed as described above, serum samples were serially diluted 3-fold down the plate, and plates left for 2 h at room temperature. Plates were washed 3 times in wash buffer and 100 μL detection antibody was added to each well (for mouse samples HRP-conjugated rabbit anti-mouse IgG (Jackson Immuno Research,West Grove, PA), for non-human primate samples HRP-conjugated goat anti-monkey IgG (Abcam, Cambridge) and incubated for 1 h at room temperature. Plates were then washed 3 times in wash buffer and incubated for 10 min in the dark with 100 μL per well of a TMB substrate solution (BD). The enzymatic reaction was stopped with 50 μL per well of 2 N H_2SO_4. Optical density was read immediately after adding stop solution on a Versamax plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm with subtraction at 570 nm. Data analysis was done using SoftMax Pro v5.4 (Molecular Devices) and the half maximum values (EC50) determined to calculate antibody titers for each sample.

3. Results

3.1. Universal helper peptide design and predicted class II coverage and reactivity

We screened candidate epitopes for in silico predicted broad HLA class II allele cross reactivity and high affinity binding using the immune epitope data base (IEDB) CD4 T cell prediction tool [24,25]. A chimeric TT/DT epitope was designed that fit these criteria. We hypothesized that inclusion of two epitopes that would induce a CD4 memory helper T cell response in vaccinated individuals may provide an advantage over individual peptides. A cathepsin cleavage site, either pmglp or kvsvr [26] was introduced between the epitopes with the prediction that it would provide more efficient processing when taken up by antigen presenting cells. Pmglp was designed to be a selective cathepsin S substrate whereas kvsvr is a less selective cathepsin S, B and L substrate. Individual DT (D) and TT (T) peptides were generated (Fig. 1A) as well as a chimeric TD peptide without a cathepsin cleavage site. In addition, two chimeric peptides containing the pmglp or the kvsvr cathepsin cleavage site (TpD and TkD respectively) were also generated. The predicted reactivity of individual and chimeric peptides to 25 MHC class II alleles, as well as predicted binding affinity, and allele frequency are shown in Fig. 1B. The combined frequency of this set of alleles is predicted to have greater than 99% population coverage [25]. The predicted consensus of several algorithms is shown, where a lower score is a predictor of higher affinity binding. Scores higher than ten are not shown. Both T and D epitopes are predicted to have high affinity binding primarily across HLA-DRB1, with some binding to DP and DQ alleles. Interestingly combining the two peptides with a cathepsin linker in some cases alters the predicted binding affinity, for example HLA-DQA1*0301-DQB*0302.

A

T	ILMQYIKANSKFIGI
D	QSIALSSLMVAQAIP
TD	IMQYIKANSKFIGIQSIALSSLMVAQ
TpD	ILMQYIKANSKFIGIPMGLPQSIALSSLMVAQ
TkD	ILMQYIKANSKFIGIKVSVRQSIALSSLMVAQ

B

Allelic variant	Allele frequency	T	D	TpD
HLA-DPA1*0201-DPB1*0101	16			
HLA-DPA1*0103-DPB1*0201	17.5			
HLA-DPA1*01-DPB1*0401	36.2			
HLA-DPA1*0301-DPB1*0402	41.6		4.14	4.42
HLA-DPA1*0201-DPB1*0501	21.7	3.53		3.55
HLA-DQA1*0501-DQB1*0201	11.3			
HLA-DQA1*0501-DQB1*0301	35.1			
HLA-DQA1*0301-DQB1*0302	19		6.69	8.29
HLA-DQA1*0401-DQB1*0402	12.8		4.36	
HLA-DQA1*0101-DQB1*0501	14.6			
HLA-DQA1*0102-DQB1*0602	14.6		0.06	2.01
HLA-DRB1*0101	5.4	4.99	9.25	4.99
HLA-DRB1*0301	13.7	3.81	5.76	3.81
HLA-DRB1*0401	4.6	2.02	7.08	2.02
HLA-DRB1*0404	3.6	1.79	2.63	1.79
HLA-DRB1*0405	6.2	4.94	7.52	4.94
HLA-DRB1*0701	13.5	0.50		0.50
HLA-DRB1*0802	4.9	0.70	4.43	0.70
HLA-DRB1*0901	6.2	0.36	1.17	0.36
HLA-DRB1*1101	11.8	3.13	3.72	3.13
HLA-DRB1*1302	7.7	1.70		1.70
HLA-DRB1*1501	12.2	3.02	8.67	3.02
HLA-DRB3*0101	26.1	8.31	6.55	8.31
HLA-DRB4*0101	41.8		1.62	4.97
HLA-DRB5*0101	16	0.07		0.07

Fig. 1. Peptide sequence and computational analysis of predicted affinity to 25 HLA Class II alleles. (A) Individual epitopes from tetanus toxoid (T), diphtheria toxoid (D), and chimeric peptides with cathepsin cleavage sites TpD (pmglp) and TkD (kvsvr), or without a cleavage site (TD). (B) Computational analysis using the Immune Epitope Data Base (IEDB) T cell epitope prediction tool for individual epitopes of chimeric epitope TpD. Lower consensus score predicts higher affinity, values >10 are not shown.

3.2. Chimeric peptide TpD shows a recall response in PBMC from multiple donors, and induces activation of cells with a CD4 central memory phenotype

We analyzed peripheral blood mononuclear cells (PBMC) from 20 individual donors for the ability to activate T cells with a CD4 memory phenotype. A representative example from one donor is shown in Fig. 2A. Individual tetanus (T) and diphtheria (D) peptides showed limited induction of CD4 + CD45RAlowCD62L+ cells expressing IFN-γ compared to a non-stimulated (NS) control (0.04% and 0.08% vs. 0.01% respectively). The chimeric peptide with no cleavage site (TD) and the peptide with the kvsvr cathepsin cleavage site (TkD) showed slightly more cells expressing IFN-γ (0.32%). However the peptide with the pmglp cathespsin cleavage site (TpD) induced a superior response (1.28%), 4-fold higher than the chimeric peptide with no cleavage site. We went on to analyze PBMC from 20 donors (Fig. 2B) and found that we could not detect a specific response in most cases using either individual T (2/20 donors) or D (7/20 donors) peptides. More donors responded to the chimeric TD peptide (15/20) but all 20 donors showed a recall response to the TpD chimeric peptide. The percentage of CD4 + CD45RAlowCD62L+ cells expressing IFN-γ normalized to a non-stimulated control for each of the peptides is shown in Fig. 2B. In addition to providing the highest percentage of responders, the TpD peptide induced the highest levels of IFN-γ among all peptides tested. Interestingly TkD had diminished activity compared to TpD, suggesting that the kvsvr cleavage site may be detrimental.

We next evaluated the type of memory cells stimulated by TpD. Central memory cells, thought to be the most effective at generating a recall response, are CD4 + CD45RAlowCD45RO + CD27 + CCR7+ [27] and express multiple cytokines including IFN-γ and TNF-α [4], whereas effector memory cells are CD4 + CD45RAlow CD45RO+/−CD27-CCR7-. Multicolor flow cytometry analysis suggested that the cells responding to TpD express a phenotype of central memory T cells (Fig. 2C). We next addressed if the memory cells favored a Th1 or Th2 phenotype upon activation. Memory T cells can be divided based on differential chemokine receptor expression into subsets that will produce either the Th1 cytokine IFN-γ, or Th2 cytokine IL-4, on activation [28,29]. We analyzed four separate donors and found that individual T and D peptides, as well as chimeric peptides induced expression of IFN-γ in more memory T cells than IL-4, suggesting a bias toward a Th1 subset (Fig. 2D). Based on these characteristics TpD was selected as the memory T helper stimulating peptide for a nanoparticle based vaccine.

3.3. TpD is required for generation of an antibody response in nicotine nanoparticle vaccinated mice

PLGA/PLA nanoparticles have been useful vehicles for vaccine development. We designed a nanoparticle vaccine carrying nicotine as the B cell antigen (Fig. 3). The components of the nanoparticle include: PLA-PEG-Nicotine, which is a block copolymer with nicotine covalently bound to the free end of PLA-PEG; the adjuvant R848 linked to PLGA, and the memory T cell helper antigen TpD (Fig. 3). To assess the contribution of TpD, nanoparticles were also generated that lacked TpD. As an initial test for efficacy, we immunized mice with nanoparticles containing or lacking TpD (Fig. 4). Mice were immunized on days 0, 14, 28 and 169. The nanoparticle containing TpD induced robust anti-nicotine antibody titers, whereas nanoparticles lacking TpD showed no detectable antibody response (Fig. 4A). Antibody levels increased with each boost, particularly after the third boost on day 169, 141 days after the previous immunization, suggesting helper T cell memory was long lived.

To further assess long-lived T cell memory, we immunized mice on days 0, 14 and 28 with nicotine nanoparticles containing R848 and either TpD or ovalbumin 323–339 (Ova) peptide (Fig. 4B). Spleens were harvested 122–152 days after final inoculation and either not stimulated, or stimulated ex vivo with TpD or Ova peptide. Supernatants were harvested after 18 h and evaluated for IFN-γ levels. In TpD immunized mice, IFN-γ secretion was not detectable when splenocytes were non-stimulated or challenged with the Ova peptide. In contrast IFN-γ was detected at significant levels when splenocytes were stimulated with TpD. Conversely, in Ova immunized mice only the Ova peptide was able to induce a response. The data suggests that TpD, when delivered in a nanoparticle, is able to provide long term CD4T cell memory and can function on re-challenge to provide a boost in a vaccine response.

In order to evaluate the dose-dependent effect of helper peptide on anti-nicotine antibody titers in vivo, we designed an experiment using limiting levels of TpD. Mice were immunized on days 0, 14 and 28, and on day 46 serum analyzed for antibody titers (Fig. 4C). Increasing the amount of TpD during immunization resulted in elevated anti-nicotine antibody titers, suggesting that the magnitude of antibody response is helper peptide dependent.

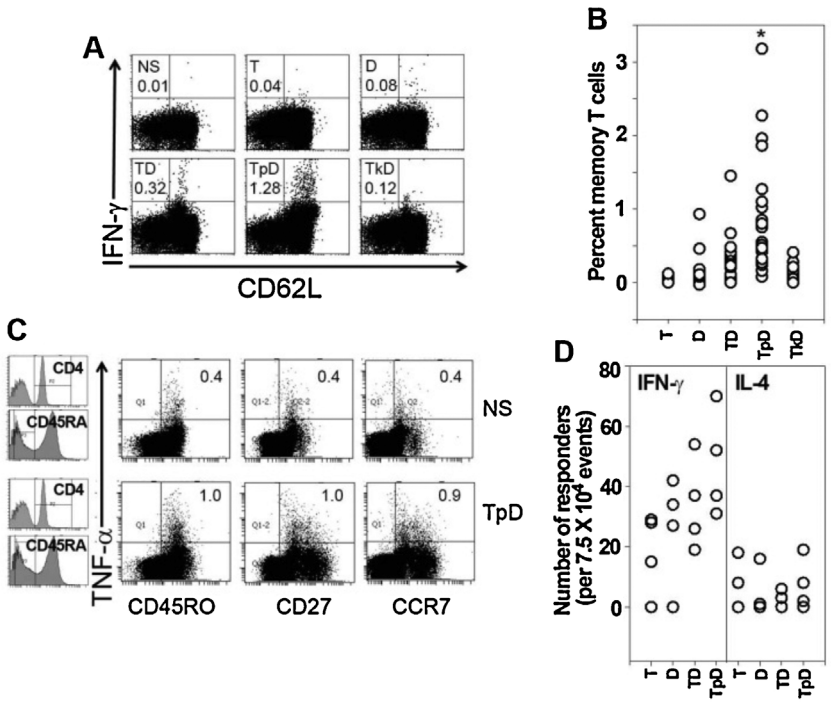


Fig. 2. Characterization of an in vitro PBMC memory T cell recall assay using flow cytometry. (A) Representative example from a single blood donor. PBMC were stimulated with different peptides overnight and then evaluated for memory T cell activation. Intracellular IFN-γ and CD62L expression, gated on CD4 + CD45RAlow cells are shown. (B) Percent memory T cells responding to various peptides indicated (% CD62L + IFN-γ+ in a CD4 + CD45RAlow gate) for 20 individual PBMC donors. (C) Further characterization of memory T cells either non-stimulated (NS), or stimulated with peptide TpD. Central memory T cells are CD4 + CD45RAlowCD45RO + CD27 + CCR7+. Activation was evaluated by staining for intracellular TNF-α. (D) Expression of Th1 (IFN-γ) or Th2 (IL-4) cytokine in central memory T cells stimulated by different peptides. CD62L + IFN-γ+ cell numbers per 7.5×10^4 CD4 + CD45RAlow cells collected by flow cytometry (events) are shown. $N = 4$ donors per group.

3.4. MHC class II peptide TpD generates a robust CD4 memory T cell recall response in nanoparticle vaccine immunized non-human primates

We further investigated TpD activity in non-human primate preclinical models. Data from rhesus monkeys immunized on days 0, 28, and 56 with escalating doses of nicotine nanoparticles are shown in Fig. 5. As expected no anti-nicotine antibody titers were seen two weeks prior to immunization or at the time of the first immunization (Fig. 5A). Antibodies were detectable after the first immunization, and increased significantly after the second and third immunization. Titers were variable at the lowest dose (0.3 mg) and plateaued at the 0.9 mg dose. Analysis of CD4 T cell recall responses showed detectable levels of TpD responding cells at the lowest dose, (Fig. 4B) but not prior to immunization. All 4 monkeys tested showed helper T cell responses. There was not a clear dose response, as expected given the small number of animals studied ($N = 1$ per group). T cell recall responses were detectable 63 days after the last immunization, suggesting memory T cells were being generated.

We next studied TpD activity in a larger cohort of cynomolgus monkeys ($N = 50$) immunized with nicotine nanoparticles and evaluated them for both anti-nicotine antibody titers and T cell recall responses (Fig. 6). Cynomolgus monkeys were pre-immunized on days −78 and −48 with a combined pediatric diphtheria/tetanus toxoid vaccine, and then immunized on days 1, 29 and 57, and 85 with the nicotine nanoparticle vaccine. Saline treated monkeys were negative for anti-nicotine titers at all time points, but all other monkeys at all doses were positive (Fig. 6A). The results showed a dose dependent escalation in antibody response plateauing at the 8 mg nanoparticle dose. The titers persisted until the last day of analysis (day 141). Peripheral blood was collected on day 85 for T cell recall analysis (Fig. 6B). Each of the ten primates dosed with 2.0, 8.0 and 16 mg of vaccine showed a positive dose escalating T cell recall response ($N = 30/30$ total) compared to saline injected controls. Additionally, 6/10 monkeys immunized with the lowest dose of 0.5 mg gave a positive recall response to stimulation with TpD (Fig. 6B). In summary, all cynomolgus monkeys immunized with the three highest doses of nicotine nanoparticles showed a positive memory T cell recall response to TpD, demonstrating that TpD was presented in vivo by cynomolgus MHC Class II molecules and generated a peptide-specific T cell recall response.

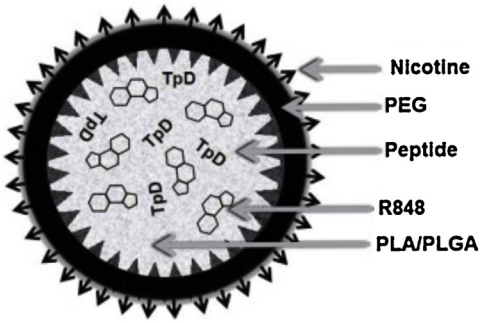


Fig. 3. Schematic representation of nicotine nanoparticle structure.

4. Discussion

Synthetic vaccines have potential advantages with respect to antigen (or epitope)-specificity, safety, and ease of manufacturing. We have recently developed a self-assembling synthetic vaccine particle (SVP) technology which enables surface display of B cell haptens, such as nicotine, and encapsulation of potent TLR agonists. The nano-sized particles directly flow through lymphatics

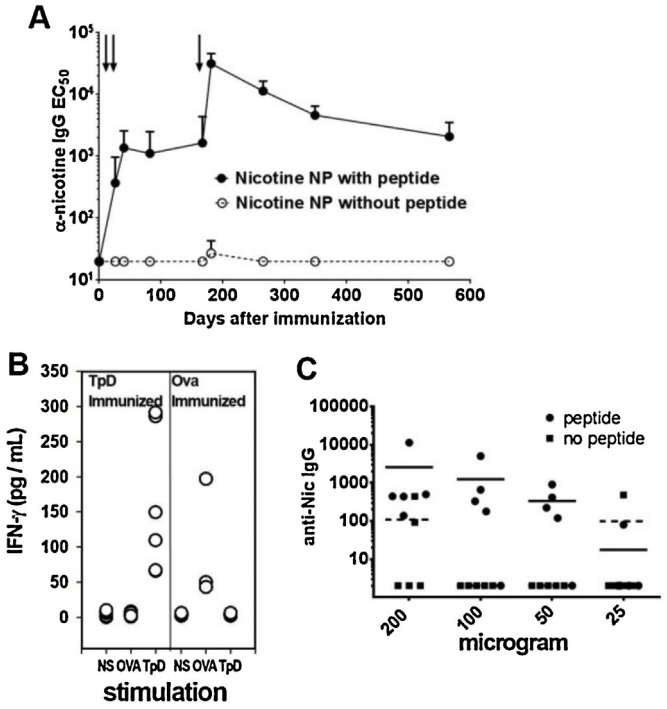
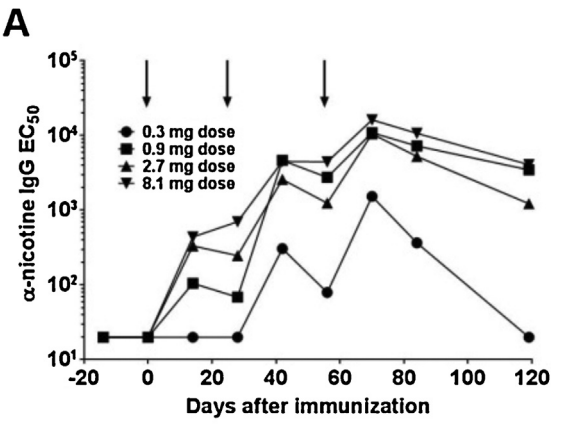


Fig. 4. In vivo evaluation of TpD peptide activity in nicotine nanoparticle immunized mice. (A) Anti-nicotine antibody titers in nanoparticle immunized mice. Mice ($N = 5$ per group) were immunized on days 1, 14, 28 and 169. Results for all dates analyzed post immunization are shown. (B) Balb/C mice were inoculated on D0, 14 and 28 with nanoparticles containing R848 and either TpD or Ova peptide. Spleens were harvested between 122 and 152 days after final inoculation, and both CD4+ T cells and CD11c+ dendritic cells isolated. Cells were incubated at a 10:1 ratio (500,000 CD4 T cells to 50,000 DCs) with 10 μM peptide: (1) specific to inoculum (2) non-specific to inoculum. Supernatants were harvested 18 h later and assayed for IFN-γ by ELISA. Number of TpD NP inoculated mice assayed = 5. Number of Ova NP inoculated mice assayed = 3. (C) Balb/C mice ($N = 5$/group), were immunized with nanoparticles containing R848 and nanoparticles containing nicotine plus TpD (peptide), or nicotine alone (no peptide). Immunizations were performed on days 0, 14 and 28, and blood collected on day 46 to evaluate anti-nicotine antibody titers.



B

Elispots per 1 X 10^6 PBMC

Day	0.3 mg	0.9 mg	2.7 mg	8.1 mg
Day 0	0	0	4	0
Day 14	1.9	23.2	31.4	31.6
Day 28	1.1	8.7	13	15.7
Day 42	117.9	220.3	106	54.4
Day 56	38.1	667.3	134	11.2
Day 70	49	240	101.5	129.1
Day 84	77.3	75.7	75.3	80
Day 119	221.1	121.6	102.3	49.3

Fig. 5. Antibody titers and memory T cell recall assays in four rhesus monkeys vaccinated with nicotine nanoparticles. (A) Blood was collected 14 days prior to immunization and then every 2 weeks after the first immunization, up to 17 weeks (day 119). Monkeys were immunized on days 1, 28, and 56 with escalating doses of vaccine at 0.3, 0.9, 2.7, and 8.1 mg/mL in 1 mL. (B) Blood was collected at the time shown (5 mL), and PBMC isolated by Ficoll centrifugation. At each time point of collection cells were plated in anti- IFN-γ coated elispot plates with or without TpD peptide. Values shown are normalized to non-stimulated controls and represent number of spots per 1×10^6 PBMC.

into lymph nodes, where they can be endocytosed and processed by APCs [30]. However a potential limitation of synthetic vaccines, and even some recombinant protein vaccines, is the lack of sufficient T cell epitopes to drive robust antibody responses. In this paper, we describe the design and demonstrate the utility of a 'universal' T cell helper peptide (TCHP) that can provide CD4 T cell help for B cell differentiation and antibody affinity maturation across a broad population.

We have taken advantage of new and improved in silico prediction tools to screen peptides for broad and high affinity MHC class II binders. This approach has proven useful for screening large numbers of potential epitopes from naturally occurring pathogen proteins, such as tetanus toxoid and diphtheria toxoid, to design better TCHPs. We created chimeric peptides based on complementary peptide epitopes that together provided broad coverage of MHC class II alleles. In order to improve the probability that a chimeric peptide would get processed properly for presentation on MHC class II protein, we included a synthetic cathepsin cleavage site between the selected TT and DT epitopes [26]. One advantage of using TT and DT derived epitopes is that most people have been previously vaccinated with DT and TT, and therefore are likely to have pre-existing T cell memory. This enabled us to validate peptide candidates by screening them for the ability to induce a memory T cell response in normal peripheral blood donors. We assessed CD4 memory T cells by flow cytometry for cell surface markers and induction of cytokine expression. We found that 20/20 donors responded to the chimeric peptide TpD with a synthetic cathepsin S cleavage site. Individual peptides alone showed fewer numbers of cells responding in fewer numbers of subjects. The frequency of responders to individual peptides (T and D, 10% and 35% respectively) was lower than that reported by others, perhaps due to the use of a different assay [3–11]. Interestingly the recall response to the chimeric peptide (TD) was greater than the sum of the response to the individual epitopes. Memory T cells can be characterized as effector or central memory cells by cell surface markers (CD4, CD45RA, CD45RO, CD27, CCR7) and cytokine expression (IFN-γ, TNF-α and IL-4) [27–29]. Central memory T cells are thought to give a faster and better response to epitope challenge than naïve T cells. Further characterization showed that the T cells responding to TpD had cell surface markers and cytokine expression consistent with central memory CD4 cells. Based on these results we selected TpD for nanoparticle vaccine formulation, and evaluation in mouse and primate animal models.

We used a fully synthetic nanoparticle vaccine against nicotine, as a model system to test the activity of the TpD peptide. Studies in mice demonstrated that TpD was both necessary and sufficient for the ability to induce a robust anti-nicotine antibody response. Nanoparticles lacking TpD induced little or no antibody production, while TpD-containing nanoparticles induced antibody titers which increased with each successive boost. In particular, a boost administered at day 169, 141 days after the last immunization, induced a 19-fold increase in antibody titer, indicating that TpD induced long term memory T cells. This was confirmed by assessment of in vitro antigen-specific T cell recall to TpD using lymphocytes from immunized mice. Positive results achieved with the mouse studies prompted us to study more relevant nonhuman primate models, initially with a small cohort of 4 rhesus monkeys, and subsequently

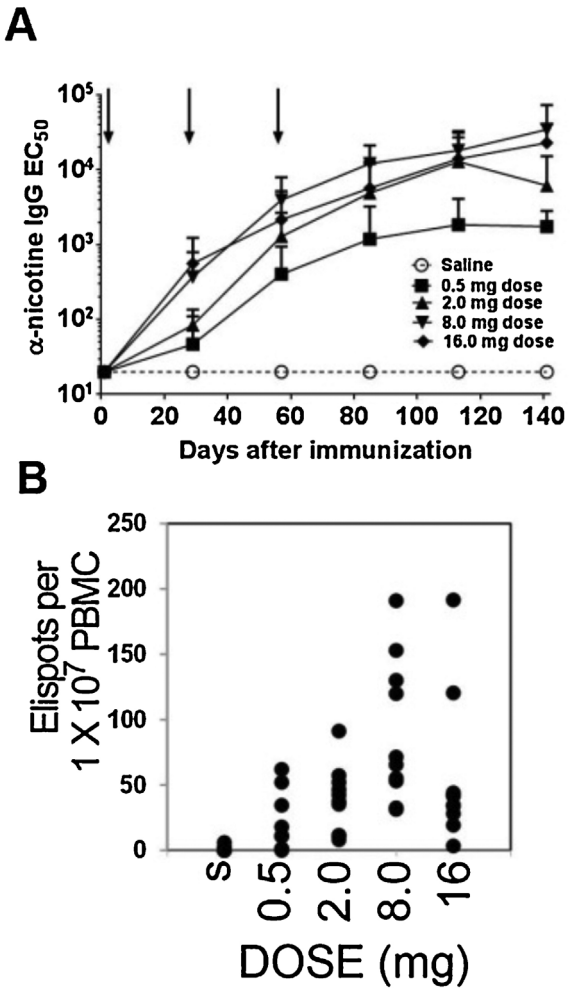


Fig. 6. Antibody titers and memory T cell recall assays in nicotine nanoparticle vaccinated cynomolgus monkeys. (A) Monkeys were immunized ($N = 10$ per group) on days 1, 29, and 57 with saline, or escalating doses of 1 mL vaccine at 0.5, 2.0, 8.0 and 16.0 mg/mL. Blood was collected on days shown, prior to immunization (day 1) and then on days 29, 57, 85, 113, and 141 to test for anti-nicotine antibodies. (B) Peripheral blood was collected on day 85 for T cell recall analysis (3 mL) and PBMC isolated by percoll centrifugation. PBMC were plated in anti- IFN-γ coated elispot plates with or without TpD peptide. Values shown are normalized to non-stimulated controls and represent number of spots per 1×10^7 PBMC.

with a large cohort of 50 cynomolgus monkeys previously immunized with a DT and TT vaccine. Both studies were designed to provide an assessment of antibody and T cell help data over an extended period of time. Monkeys were from an outbred population, so their MHC class II alleles are variant and therefore a good model to test the 'universality' of TpD. Rhesus monkeys immunized with the nicotine nanoparticle produced sustained antibodies in a dose-dependent fashion, and T cell recall for over 4 months. The cynomolgus monkeys also showed a robust and dose dependent antibody response to a nicotine nanoparticle vaccine. All cynomolgus monkeys (30/30) immunized with the three highest doses of vaccine showed a positive memory T cell recall response to TpD, while those immunized with the lowest dose showed 6/10 responders. It is likely that the low responder numbers at the lowest dose was a function of dose rather than MHC class II allele distribution.

Alexander et al. described a de novo designed non-natural pan-DR epitope peptide (PADRE) that binds promiscuously to common HLA-DR alleles [2]. The PADRE peptide has been tested in a number of clinical trials. BCR-ABL peptides linked to PADRE and co-administered with GM-CSF to patients with chronic myeloid leukemia elicited a PADRE-specific recall response in 14 of 14 subjects tested [31]. PADRE peptide admixed with MAGE3 peptide in incomplete Freunds adjuvant administered to melanoma patients elicited detectable but low levels of PADRE-reactive effector cells in 7 of 9 subjects [32]. PADRE peptide and WT-1, Muc-1, and proteinase-3 CTL epitopes admixed with CpG oligonucleotides in montanide and administered to patients with acute myeloid leukemia and multiple myeloma induced an increase in PADRE-reactive effector T cells in all subjects, although these T cells showed an apparent defect in IL-2 secretion [33]. In contrast, a DNA vaccine encoding 21 HIV-specific CTL epitopes and PADRE was tested in 42 healthy volunteers and elicited only one positive recall response to PADRE as measured by ELISpot [34]. Finally, autologous dendritc cells pulsed with the PADRE elicited an ex vivo recall response to PADRE in 10 of 18 subjects in one study [35] and low level responses in another study [36]. Not surprisingly, the efficacy and universality of the PADRE peptide may be dependent upon the context in which the peptide is administered, such as dose, regimen, route, adjuvant, and form (free peptide, linked peptide, DNA-encoded, or pulsed DCs). One of the potential advantages of using a universal T cell helper peptide based on TT and DT is that pre-existing CD4 T cell memory to TpD from prior immunization with DT and TT may confer an advantage for a TpD-containing nanoparticle vaccine by generating a larger pool of antigen-specific T cells that can provide faster and more efficient help to B cells in a secondary challenge [37–39].

In addition CD4 memory T cells have several functional characteristics that facilitate a more robust response to antigen. For example, CD4 memory T cells have a lower threshold for activation by antigen than naïve cells and show polarized differentiation to specific T cell subsets (e.g. Th1, Th2, Th17, and T follicular helper (Tfh) subsets), and multi-cytokine expression (e.g., TNF-α, IL-2 and IFN-γ) [40]. In particular, CXCR5 expressing memory CD4 cells have been found to provide accelerated help to B cells, perhaps due to their ability to localize to B cell follicles [41]. Overall the data suggests that the existence of CD4 memory T cells will be beneficial in producing a more rapid and robust induction of antibody production. As a result there may be an advantage in targeting memory T cell activation to enhance a response in vaccines.

It was originally hypothesized that conjugate vaccines would benefit from pre-existing memory against the carrier, but surprisingly, it was found that prior vaccination with the carrier (e.g. TT or DT) actually inhibited the antibody response to the hapten conjugate [42]. This phenomenon, termed epitope-specific suppression or epitopic suppression [42–45], also extends to haptens conjugated to virus-like particles [46]. While both T cells and B cells have been implicated in the mechanism of epitope-specific suppression, the inhibitory effect appears to be largely due to competition with pre-existing carrier-specific B cells and antibodies [47]. Importantly, epitope-specific suppression observed with gonadotropin releasing hormone (GnRH) peptide conjugated to DT could be bypassed by conjugating GnRH to a T cell helper peptide derived from DT [48]. These results suggest that epitope suppression is restricted to memory B cell epitopes not memory T cell epitopes. Thus we expect that that nanoparticle vaccines containing TpD peptide would have the benefit of leveraging pre-existing CD4 memory T cells without invoking B cell-mediated epitope-specific suppression.

In conclusion we have developed a chimeric MHC class II memory recall peptide, TpD that gives broad MHC class II coverage in humans, and is potent in generating a recall response in mice and non-human primates. It is possible that this will be a valuable tool for providing enhanced responses against poorly immunogenic vaccines.

Conflict of interest: All authors are employees and shareholders of Selecta Biosciences.

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