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The Human Immune System Recognizes Neopeptides Derived from Mitochondrial DNA Deletions

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Mutations in mitochondrial (mt) DNA accumulate with age and can result in the generation of neopeptides. Immune surveillance of such neopeptides may allow suboptimal mitochondria to be eliminated, thereby avoiding mt-related diseases, but may also contribute to autoimmunity in susceptible individuals. To date, the direct recognition of neo-mtpeptides by the adaptive immune system has not been demonstrated. In this study we used bioinformatics approaches to predict MHC binding of neopeptides identified from known deletions in mtDNA. Six such peptides were confirmed experimentally to bind to HLA-A*02. Pre-existing human CD4+ and CD8+ T cells from healthy donors were shown to recognize and respond to these neopeptides. One remarkably promiscuous immunodominant peptide (P9) could be presented by diverse MHC molecules to CD4+ and/or CD8+ T cells from 75% of the healthy donors tested. The common soil microbe, Bacillus pumilus, encodes a 9-mer that differs by one amino acid from P9. Similarly, the ATP synthase F0 subunit 6 from normal human mitochondria encodes a 9-mer with a single amino acid difference from P9 with 89% homology to P9. T cells expanded from human PBMCs using the

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Abbreviations used in this article: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CADV, empty adenovirus; CD80-AdV, adenovirus containing CD80; FI, fluorescence index; FMO, fluorescence minus one; h, human; MFI, mean FI; mt, mitochondrial; SFU, spot-forming unit; WT, wild-type.

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mutations lead to higher expression of MHC class I molecules in human cells, and β2-microglobulin-deficient mice have higher accumulation of mt mutations than do WT mice (16), providing indirect evidence of immune surveillance of mt mutations. The significantly higher mtDNA mutation load in rheumatoid arthritis patients compared with osteoarthritis patients is among the mounting evidence that neopeptides generated from mutated mtDNA have a role in autoimmune disease (17). The presence of large-scale deletions in mtDNA in multiple sclerosis patients also adds weight to this hypothesis (18). However, to date the direct recognition of such neopeptides by the immune system has not been reported.

In this study, to our knowledge we show for the first time that neopeptides from mtDNA harboring large-scale frameshift deletions bind to MHC molecules and can be recognized by T cells from normal individuals, providing strong evidence for immune surveillance of altered mitochondria, which in turn may function to limit the accumulation of damaged mitochondria with age. Alternatively, the close similarity of one such mutated mt peptide to both a self-peptide and an environmental microbe also leads us to hypothesize that this immune surveillance mechanism can result in autoimmunity in susceptible individuals.

Materials and Methods

Identification of mutations

Human mtDNA sequences were obtained from the National Center for Biotechnology Information (reference sequence ID NC_001807). Deletion mutations in the coding regions of CO1, ATP6, and ND4 were identified using http://www.mitomap.org (19).

Software used

The MATLAB Bioinformatics Toolbox was used for sequence manipulations, concatenations, and gene assembly. SYFPEITHI is a database comprising >7000 known MHC class I and II binding peptide sequences compiled from published reports. SYFPEITHI was used to identify the 9-mers based on their potential to bind to HLA-A*0201 (20).

NetChop produces neural network predictions for cleavage sites of the human proteasome and was used to identify proteasomal cleavage sites in the polypeptides identified in the mtDNA-containing deletion mutations (21). RankPep predicts peptides that bind to MHC classes I and II from protein sequence or sequence alignments using position-specific scoring matrices. RankPep predicts those MHC class I peptide ligands with a C terminus that is likely to be the result of proteasomal cleavage. It was used to identify potential peptides with C-terminal sequences appropriate for binding and presentation (22).

NetMHCpan predicts binding of peptides to any known MHC using artificial neural networks. The artificial neural networks of NetMHCpan were trained on >115,000 quantitative binding data covering >120 MHCs. Eighty-six percent of NetMHCpan predictions have been experimentally confirmed (23).

NetMHCII 2.2 predicts binding of peptides to MHC class II alleles using artificial neural networks. Predictions can be obtained for 14 HLA-DR alleles (covering the nine HLA-DR supertypes), 6 HLA-DQ, and 6 HLA-DP alleles (23).

Peptide synthesis

Eighteen neopeptides derived from altered mtDNA sequences, the influenza matrix protein-derived peptide (GLILGFVFTL) and HIV-prog p17 (SLYNTVATL) were synthesized by Proimmune (Sarasota, FL) at the recommended purity for T cell assays. Aliquots were stored at −80°C.

MHC/peptide binding

MHC stabilization assays were performed with T2 cells to test the predicted HLA-A2 binding of the neopeptides. T2 cells are TAP-deficient and thus have few class I MHC proteins on their surface (24, 25). Addition of exogenous peptides stabilizes MHC class I on the surface and can be used as evidence of peptide binding. TAP-deficient T2 cells were cultured in complete medium containing RPMI 1640 (Life Technologies) supplemented with 10% FBS, 0.1% 2-ME, 1× nonessential amino acids (Life Technologies), 1 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics. Cells were incubated in 5% CO2 at 37°C conditions. T2 cells suspended in complete medium were distributed at 2 × 105 per well in a 96-well flat-bottom plate and incubated with each neopeptide overnight at varying concentrations. T2 cells were then washed once in FACS buffer (PBS with 1% [w/v] BSA and 0.1% [w/v] sodium azide) and stained with FITC-labeled anti–HLA-A2 Ab for 30 min at 4°C. The cells were washed twice with FACS buffer, and MHC stabilization was determined using a FACS Calibur. Influenza peptide-treated cells served as a positive control, while T2 cells without peptide were a negative control. The fluorescence index (FI) was calculated as: FI = (MFI sample − MFI background)/MFI background, where MFI is mean FL, and background is the negative control value. FI is the average of at least two independent experiments.

Recruitment of human donors and blood collection

The Human Participants Review Board and the Biosafety Committee of York University (Toronto, ON, Canada) approved these studies. Twelve healthy human donors between the ages of 22 and 66 y were recruited and provided informed consent. No donor had any recent illnesses (in the past 6 mo) and no donor had any history of one or more of autoimmune diseases. Approximately 20 ml venous blood was drawn using the BD Vacutainer Safety-Lok blood collection set with a preattached holder (Source Medical Corporation). At the same time, ~20 μl blood was obtained using BD Microtainer contact-activated lancets (Source Medical Corporation) for the QIAcard FTA spots (Qiagen, Toronto, ON, Canada) and, after drying, were stored in pouches at room temperature.

PBMC isolation and storage

Immediately after blood draw, PBMCs were purified using Ficoll-Hypaque gradient centrifugation followed by cryopreservation in liquid nitrogen using 40% complete medium, 50% endotoxin-free FBS, and 10% DMSO.

HLA typing

Olerup (Westchester, PA) sequence-specific primer PCR HLA-A and HLA-DR low-resolution kits were used to determine HLA type. Punches from FTA discs were used directly, PCRs with primer sets specific for each allele were performed and the HLA type was determined by the presence and relative sizes of bands after electrophoresis in 0.8% agarose gels using lot-specific specificity tables.

IFN-γ ELISPOT assay

ELISPOT assay kits (human IFN-γ ELISPOTPRO kits, Mabtech, Nacka Strand, Sweden) were used to determine the frequency of epitope-specific IFN-γ-secreting T cells. PBMCs (2 × 105well/100 μl) were pulsed with peptides (10 μM) overnight in polyvinylidene difluoride plates precoated with anti-human IFN-γ mAb at 37°C in 5% CO2. After overnight incubation, wells were washed with filtered PBS to remove the non adherent cells. Following washing, each well was treated with 100 μl one-step detection reagent (alkaline phosphatase–conjugated detection IFN-γ Ab) at 1:200 in filtered PBS containing 0.5% FBS for 2 h at 37°C. Plates were washed five more times with filtered PBS before chromogenic development with 100 μl/well ready-to-use 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/NBT. BCIP/NBT-added plates were incubated for 15 min at room temperature, during which time the blue spots developed. Each spot-forming unit (SFU) was counted to one SFU = γ-IFN-secreting T cell. The numbers of Ag-specific T cells are presented as SFU per total number of PBMCs added. The negative control was PBMCs in medium without peptide stimulation and was used to assess the spontaneous secretion of IFN-γ. To verify that the ELISPOT was working, we used the polyclonal activator anti-CD3 mAb as a positive control with each donor. Each peptide was incubated overnight in ELISPOT plates without PBMCs to detect nonspecific reactions. The negative control was PBMCs in medium without peptide stimulation and was used to assess the spontaneous secretion of IFN-γ. A well was considered positive when it contained at least 10 SFU and twice as many SFU as the negative control wells. Each datum point is the mean of duplicate wells.

T cell costimulation assay using CD80-modified monocytes as APCs

T cells were expanded with peptide and costimulation, as previously described (26). Briefly, PBMCs were suspended at 7.5 × 105 cells/ml and plated at 0.1 ml/well in 96-well plates. Adherent monocytes were enriched at 37°C for 1 h and then infected with adenovirus containing CD80 (CD80-Adv) or empty adenovirus (CADV) overnight. Nonadherent cells were removed and stored overnight at 37°C to be used as a source of T cells. CD80-Adv or CADV was added to the adherent monocytes at a multiplicity of infection of 100, followed by centrifugation at 3000 rpm at 37°C for 1 h.
The no Ag control cultures included monocytes treated with CD80-Adv alone. The neopeptides were added to the CD80-Adv and CDAdV-modified monocytes at 10 μM. Following overnight incubation, the cultures were removed to wash excess AdV and peptide. T cells were purified from the nonadherent cell fraction by magnetic negative selection using Pan T cell isolation kit II from Miltenyi Biotech (MACS). T cells at 2 × 10^6 cells/ml were added to the activated monocytes and coincubated at 37°C for 7 d. For the Ab blocking experiments, adenovirus-modified monocytes were incubated with W6/32 (MHC class I blocking) Ab or L243 (HLA-DR blocking Ab) or HLA-DP blocking Ab (clone B7.21) at 10 μg/ml for 1 h prior to the addition of peptide. On day 8, expanded T cells were restimulated with 5 × 10^5 peptide for 6 h in the presence of GolgiPlug (BD Biosciences), a protein transport inhibitor. Restimulated cells were first stained for surface markers, fixed, and permeabilized using the Cytofix/ CytoPerm kit (BD Biosciences), followed by intracellular cytokine staining. Cell fluorescence was measured using a FACS Calibur and were analyzed with FlowJo software (Tree Star, Ashland, OR). The following mAbs were used: human (h)-specific anti-hCD3, -hCD8α, -hCD4, and –hIFN-γ (eBioscience). In all figures, fluorescence minus one (FMO) was used to determine background levels of staining. Approximately 1 × 10^2 to 4 × 10^3 total T cell events were collected per condition per donor.

**Ex vivo tetramer staining assay**

MHC tetramers were provided by the National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA). Tetramers were custom made for HLA-A*0201 with the P9 neopeptide (YVFITLVPVL), the influenza matrix protein-derived peptide (GILGFVFTL), and HIV-gag p17 (SLYNTV ATL). T cells were cultured with peptides as described in the costimulation assay. On day 8, cells were first stained with the tetramer for 15 min at 37°C followed by staining for surface markers with anti-hCD3 and anti-hCD8α (eBioscience) at 4°C for 20 min. In all figures, FMO was used to determine background levels of tetramer staining. FlowJo software (Tree Star) was used for data analysis. Approximately 1 × 10^2 to 4 × 10^3 total T cell events were collected per condition per donor.

**Tetramer staining assay to determine the original phenotype of P9 neopeptide tetramer+CD8+ T cells**

PBMCs were thawed and rested for 2 h at a density of 2 × 10^6 cells/well in a round-bottom microtiter plate. Tetramers used were custom made for HLA-A*0201 with the YVFITLVPVL. T cells were cultured with peptides as described in the costimulation assay. On day 8, cells were first stained with the tetramer for 15 min at 37°C followed by staining for surface markers (anti-hCD3, -hCD8α, -hCD45RA, and hCD27; eBioscience) at 4°C for 20 min. In all figures, FMO was used to determine background levels of tetramer staining. FlowJo software (Tree Star) was used for data analysis.

**Statistical analysis**

Unpaired Student t tests were performed using GraphPad Prism software (GraphPad Software, San Diego, CA) to compare T cell responses. A p value of <0.05 was considered significant.

**Results**

**Identification of peptides predicted to bind to the MHC HLA-A*0201 allele**

To identify potential immunoreactive neopeptides, we used Mitomap as described in Materials and Methods. Of the 116 deletion mutations identified, 101 were selected based on the criteria that the deletions were within an open reading frame and that the fused sequences could be expressed and translated. The sequences were concatenated at the deletion points, and 300 nt flanking each side of the deletion breakpoint, including the mutation site, were computationally translated in the normally used reading frame using the mammalian mt genetic code in the MATLAB Bioinformatics Toolbox. Neopeptides were further screened for their absence in any of the 13 mitochondrially encoded native protein sequences as well as for their predicted binding to a class I MHC allele (using bioinformatics-based methods described in Materials and Methods and in Fig. 1). The 101 translated sequences were screened with SYFPEITHI to identify 9-mers that had the potential to bind to HLA-A*0201, the initially selected test allele. For each mutated mtDNA sequence, neopeptides with an SYFPEITHI score of at least 24 were selected as being potentially strong binding affinity neopeptides (27) and were subsequently assessed for proteosomai cleavage probability using NetChop and RankPep (detailed in Materials and Methods). Based on these criteria, 18 neopeptides were chosen for experimental testing. Characteristics of the neopeptides identified from the mutant mtDNA are shown in Supplemental Fig. 1. Full details on these mutations are listed in Supplemental Table 1. The SYFPEITHI scores of each neopeptide are shown in Fig. 1A.

**Identification of HLA-A2–stabilizing neopeptides**

To determine whether the 18 predicted neopeptides bind to HLA-A*02, their ability to stabilize the HLA-A*02 molecule on Ag-processing–deficient T2 cells was evaluated (described in Materials and Methods). Influenza matrix protein-derived peptide (58GILGFVFTL66; SYFPEITHI score of 30) was used as a positive control. Six of the 18 neopeptides (P4, P9, P10, P16, P17, and P18) demonstrated dose-dependent HLA-A*02 stabilization on T2 cells. The remaining 12 neopeptides displayed minimal effect on HLA-A2 levels and/or failed to show dose-dependent binding.

**Healthy human IFN-γ T cell memory responses to neopeptides**

T cell responses in PBMCs of 12 healthy individuals were tested by overnight stimulation with synthesized neopeptides using the IFN-γ ELISPOT assay (Supplemental Table II shows the details of donors). Because naive T cells cannot be activated to produce IFN-γ within the 18–24 h stimulation time frame, this overnight incubation time ensures that the immune response to these neopeptides is from pre-existing memory T cells in the donors. Fig. 2 shows the response to peptides, divided into A22 and A26 donors as well as into older and younger donors. All four of the older (>60 y) A26 donors showed a response of >10 SFU/200,000 cells to at least one of the peptides, with several peptides inducing responses of >40 SFU/200,000 cells in at least one of the older donors (Fig. 2A). Donor OA1 (66 y of age) responded to 6 of 18 neopeptides (33%) tested. Two neopeptides, P9 and P13, stimulated IFN-γ responses in PBMCs from other older donors. Alternatively, little or no response to the neopeptides was observed in the younger HLA-A*026 donors (mean age of 23.75 y) with the exception of neopeptide P9, which induced a weak response in two younger donors (Fig. 2A). Analysis of age versus frequency of IFN-γ responses to P9 showed a positive correlation with age, although not statistically significant (Supplemental Fig. 2). Although a larger sample size would be required to confirm an age-related response, the results are consistent with T cell responses to neopeptides accumulating with age.

**Promiscuous MHC class I and II restriction of the neopeptides**

HLA-A*026 donors (mean age of 42 y) also responded to some of the identified neopeptides (Fig. 2B). T cells from donor NA1 (34 y of age) responded to 6 of 18 neopeptides tested, albeit at lower magnitude when compared with the HLA-A*026 older donor OA1. Other HLA-A*026 donors had T cells with detectable responses to P9 and P13. Because T cells from HLA-A*026 donors showed responses to the HLA-A*02 binding neopeptides, bioinformatics prediction and HLA typing was used to evaluate the possible restriction of these reactive neopeptides to HLA alleles other than A*02. The analysis revealed that the predicted MHC restriction of neopeptides P9 and P13 matched the HLA-type of A*026 donors NA1 and NA2, likely explaining their response to these peptides (Table I). When neopeptide P9 was screened for its potential binding to HLA-B and
HLA-C alleles using the NetMHCpan server, it was predicted to strongly bind to a number of HLA-B and HLA-C alleles (Table II). Additionally, using the NetMHCII 2.2 server, neo-peptide P9 was predicted to be presented by class II alleles HLA-DRB1*0101, as well as HLA-DPA1*03:01-DPB1*04:02, HLA-DPA1*01:03-HLA-DPB1*03:01, and HLA-DPA1*01:03-HLA-DPB1*04:01 alleles, albeit with modest predicted binding affinity. These results suggest that some neopeptides could bind promiscuously to several MHC molecules, including restriction of one of the neopeptides (P9) to both an MHC class I and a class II allele.

Phenotype of T cells responding to neopeptide P9 in healthy donors

Because peptide P9 was immunogenic in most of the donors (with 75% of donors showing a response in the ELISPOT assay), we further evaluated this response. T cells from HLA-A*02+ and HLA-A-02 P9-responsive donors were expanded for 7 d in the presence of neopeptide P9 and costimulation. On day 8, T cells were briefly restimulated with peptide and the IFN-γ-secreting cells were analyzed by flow cytometry. The assays demonstrated that both CD3+CD8+ and CD3+CD4+ T cells from donors OA1 and NA1 responded to neopeptide P9 (Fig. 3A and 3B, respectively).

To confirm that the response was MHC-specific, we included the pan-MHC class I-reactive Ab W6/32 in the cultures to block MHC class I recognition. Indeed, W6/32 addition greatly reduced the expansion of CD3+CD8+ T cells in response to P9, whereas, as expected, the CD4+ T cell response to this peptide was not blocked (Fig. 3). Alternatively, the anti-DR Ab L243 did not significantly reduce the CD4+ T cell response to these peptides is DP-restricted. Treatment of APCs with class II HLA-DR blocking Ab (clone B7.21), prior to the addition of neopeptide P9, resulted in significant reduction in the expansion of CD3+CD4+ T cells in response to P9 (Fig. 4). This observation confirms the potential class II HLA restriction of CD3+CD4+ T cells recognizing P9. Taken together, these data confirm the MHC class I and II–restricted recognition of P9 in both A*02+ and A*02 donors and provide evidence that P9 can be promiscuously presented to both CD4+ and CD8+ T cells.

T cells expanded by P9 peptide bind A*02/P9 tetramers

To further verify that the T cells in the cultures were responding specifically to the neopeptide P9, we analyzed the binding of expanded T cells to HLA-A*02 tetramers containing the P9 peptide. The tetramer specificity was verified using PBMCs from an HLA-A*02 donor (NA1) (Fig. 5). CD3+CD8+ T cells expanded using P9 peptide reacted with the P9/A*02 tetramer (1.59%) whereas there was minimal tetramer binding to T cells expanded from a young healthy donor (YA3) who was nonresponsive to P9 in the IFN-γ ELISPOT assay (0.422%), and there was minimal tetramer binding to T cells from an A2-2 donor (NA1, 0.282%). Moreover, P9-expanded T cells did not bind an HIV-gag p17 (SLYNTVATL)/A*02 tetramer. Thus, healthy donor OA1 has pre-existing memory CD8+ T cells.
CD8+ memory T cells recognize neopeptide P9

Having established the tetramer specificity of HLA-A*02 tetramer of P9, we next sought to characterize the surface phenotype of P9 T cells immediately ex vivo to determine whether the tetramer reactivity was due to naive or Ag-experienced cells. The expression of the leukocyte common Ag isoform, CD45RA, as well as the costimulatory molecule CD27, has been used to characterize CD8 T cell subsets. The CD45RA−CD27+ subset of CD8 T cells is thought to represent the naive compartment whereas the memory compartment is represented by CD45RA−CD27+ (central memory cells), CD45RA−CD27+ (effector memory T cells), and CD45RA+CD27− (highly differentiated effector memory population) cells, respectively (28–30).

P9/A*02 tetramer+CD8+ T cells largely consisted of CD45RA+CD27+ memory cells, with most exhibiting a CD45RA+CD27+ memory phenotype as shown in Fig. 6. These results support the conclusion that P9-reactive cells in donor OA1 consist of Ag-experienced T cells with a memory phenotype.

Homology of neopeptides with microbe-specific proteins and human self-proteins

Most donors tested (75%, 9 of 12; including HLA-A*02 and HLA–non-A*02) showed reactivity to neopeptide P9 in the ELISPOT assay and a substantial proportion of this reactivity was due to CD27+memory T cells. How could such reactivity arise? Reactivity could be due to an initial recognition of mutant mt peptides and the ensuing maintenance of memory. The reactivity could also be the result of priming of T cells with peptides derived from environmental organisms. We searched for homology to P9 within microbes (using BLASTP, National Center for Biotechnology Information). Remarkably, neopeptide P9 has 89% similarity with Bacillus pumilus fructose PTS peptide, YLVFTLLVPL (P9: YVFTLLVPL), differing only at position 2, with an SYEPEITHI score of 30 for HLA-A*02.

B. pumilus is a soil-dwelling, Gram+, spore-forming bacterium and has been found as a bloodstream pathogen during infancy (31, 32). A homology search with human self-protein also revealed that neopeptide P9 shares 89% similarity with mt self-protein, ATP synthase F0 subunit 6 (YVFTLLVSL; P9: YVFTLLVPL), again differing at only one amino acid and with an SYEPEITHI score of 26.

This analysis raises the possibility that P9 peptide–specific memory T cells could have been initially primed via cross-reactive responses to bacterial peptides. The findings also raise the possibility that T cells activated by a neopeptide could also cross-react with the corresponding host self-proteins.

Healthy human IFN-γ T cell memory responses to self and bacterial homologs of neopeptide (P9)

The PBMC T cell responses of the HLA-A*02 OA1 healthy individual (66 y of age) were evaluated by overnight stimulation with the synthesized self-peptide and the bacterial homolog peptide of neopeptide (P9) using the IFN-γ ELISPOT assay. This

![Graph](image-url)

**FIGURE 2.** Response of pre-existing memory T cells to neopeptides in IFN-γ ELISPOT assay. (A) HLA-A*02+ older donors and HLA-A*02+ younger donors. (B) HLA-A*02− donors. T cell memory responses were determined in the IFN-γ ELISPOT assay by overnight incubation of donors’ PBMCs with peptides in anti-IFN-γ-coated microtiter plates followed by treatment with anti–IFN-γ detection Ab conjugated to alkaline phosphatase followed with substrate BCIP/NBT treatment. Each blue spot on microtiter plates corresponds to one IFN-γ–secreting T cell (SFU). The positive control polyclonal activator anti-CD3 mAb was used in the functionality of the immunoassay. A well was considered positive when it contained at least 10 SFU and twice as many as SFU as the negative control wells. ELISPOT assays were set up in duplicate. All data are expressed as means ± SD.

Table I. Comparison of HLA-A allele of donor with the HLA-A allele predicted for peptides P9 and P13 using the NetMHCpan server

<table>
<thead>
<tr>
<th>Donor</th>
<th>MHC-I (HLA-A by Typing)</th>
<th>MHC Class I Allele Predicted for Peptide P9 (Binding Affinity in nM)</th>
<th>MHC Class I Allele Predicted for Peptide P13 (Binding Affinity in nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA1</td>
<td>HLA-A*32</td>
<td>HLA-A*32:02:01 (42.4)</td>
<td>HLA-A*32:05 (74.6)</td>
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<tr>
<td></td>
<td></td>
<td>HLA-A*32:05 (10.4)</td>
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<td></td>
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<td>HLA-A*32:08 (39.8)</td>
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<tr>
<td></td>
<td></td>
<td>HLA-A*32:12 (42.4)</td>
<td></td>
</tr>
<tr>
<td>NA2</td>
<td>HLA-A*01</td>
<td>HLA-A*31:07 (167.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLA-A*31</td>
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</tbody>
</table>

Binding affinity thresholds of peptides/MHC alleles: strong, ≤50.0 nM; weak, >50.0 to ≤500.0 nM.
short incubation time ensures that measured responses are recall responses from memory T cells. IFN-\(\gamma\) T cell response was measured as number of SFU, where each spot corresponds to a T cell. PBMCs stimulated with self-peptide and bacterial homolog peptide of P9 resulted in positive IFN-\(\gamma\) T cell responses (47.5 SFU/200,000 PBMCs, SEM 3.227 and 15.25 SFU/200,000 PBMCs, SEM 1.25).

The bacterial homolog peptide was able to generate a T cell response, which was perhaps not unexpected given the fact that it is a foreign peptide. More surprising, however, was the finding that the self-peptide generated T cell responses. This result demonstrates that there were pre-existing T cells in the peripheral blood that recognized self-peptide from mtDNA. OA1 donor also responded to P9 and was shown to have P9-specific T cells based on tetramer binding. This observation raises a testable hypothesis of cross-reactivity among P9 and its self and the bacterial homolog.

**T cells expanded by self and bacterial homologs of neopeptide P9 peptide bind A*02/P9 tetramers**

To determine whether an immunological cross-reactivity exists among P9 and its homolog peptides, additional stimulation assays were carried out. T cells from donor OA1 were expanded in the presence of self and bacterial homolog peptides, respectively, and subsequently stained with the P9-specific tetramer. T cells expanded with the self and bacterial homolog peptides showed staining with P9-specific HLA-A*02 tetramer (Fig. 7). This observation led to the conclusion that T cells recognizing self and bacterial homologs of P9 can cross-react with neopeptide P9-expressing APCs.

These data provide proof of principal that mitochondria neopeptides can be recognized by the human immune system and raise the possibility that this recognition could be initially due to molecular mimicry and could ultimately lead to recognition of the homologous self-peptide.

**Discussion**

Human health, in large part, is the result of a functional and balanced immune system, an immune system that surveys pre-
sented epitopes and eliminates pathogens while minimizing harmful autoimmune activation. The combined bioinformatics/experimental investigation presented in this study provides strong evidence that neopeptides generated from large-scale deletion mutations in mtDNA can be presented in an MHC-restricted but promiscuous fashion, and can elicit recall T cells responses from healthy individuals. Collectively, this study provides a new paradigm of immune recognition of mt neopeptides, namely, that altered mitochondria are subject to immune surveillance. The close similarity of one of the peptides to an epitope from nonmutated mt proteins raises the possibility that mt neopeptides could contribute to autoimmune diseases.

One of the tested peptides, neopeptide P9, was recognized by 75% of the healthy donors tested, with a trend toward more robust responses in older donors. P9 was promiscuous in its restriction, as it was presented to CD8+ T cells by HLA-A*02 and other MHC I alleles, as well as stimulating CD4+ T cell responses. The promiscuity of P9 notwithstanding, the question remains as to how pre-existing immunity to P9 (and other neopeptides) arose in the healthy donors. Bioinformatics analyses and experimental investigations identified possible sources. The common soil microbe, B. pumilus, has an encoded 9-mer that differs by one amino acid from P9. Similarly, the ATP synthase F0 subunit 6 from normal human mitochondria encodes a 9-mer with a single amino acid difference from P9. 

The high frequency of responses in this study suggests that mt mutations and their recognition by the human immune system are frequent occurrences. However, we were unable to detect mtDNA with these deletion mutations in the blood of healthy individuals by PCR amplification of mtDNA sequences (including OA1; data not shown). Although other tissues might harbor higher levels of mutated mitochondria, the lack of detection of these mutations in healthy individuals is consistent with their rapid elimination by the immune system. Because T cells are exquisitely sensitive to low
numbers of MHC/peptide complexes (57–59), it may take very few mutant mitochondria to initiate an immune response. Based on IFN-γ T cell responses detected after overnight stimulation with neopeptides, there appeared to be pre-existing T cell responses to neopeptide P9. Consistently, CD8⁺T cells for P9 were predominantly of a CD45RA⁺memory phenotype. Mitochondrial mutations accumulate with age (60). It is plausible that exposure to neopeptide P9 arising from mt mutations in the older donor (OA1, 66 y of age) led to the induction of the P9-reactive T cells. A small fraction of the P9 tetramer-bright cells was of the CD45RA⁺CD27⁺"naive" phenotype. Although it is possible that this represents the naive repertoire, it has also been reported that CD45RA⁺CD27⁺ T cells from older subjects include a non-naive compartment as shown by the presence of CD11a⁺CD127⁻cells in this subset of T cells (61). Regardless, it is clear that >80% of the P9-reactive T cells has a phenotype consistent with prior activation.

The frequency and magnitude of T cell memory responses toward mt neopeptides showed a weak, but statistically nonsignificant correlation with age. Such a correlation would be consistent with other studies demonstrating the accumulation of mt mutations with age; however, further studies with a larger number of donors would be required to substantiate this conclusion.

The fact that T cell memory responses to mt neopeptides are from healthy individuals suggests a scenario whereby a robust immune surveillance mechanism is functioning to detect neopeptides arising from mutant mtDNA. Surveillance would play a significant role in preventing the accumulation of cells with inefficient mitochondria, which have the potential to lead to mt-related diseases. The robustness of the immune system in the recognition of mtDNA neopeptides is exemplified by the demonstration of MHC promiscuity and CD8⁺, CD4⁺ T cell responses toward these peptides. A single frameshift deletion can lead to many potential immunogenic neopeptides. A continuous immune response to these mtDNA neopeptides could result in and/or perpetuate an ongoing chronic inflammation state, thus leading to considerable damage to affected and unaffected tissues. The inflammation could in turn cause damage to mtDNA, resulting in a vicious cycle of increased production of reactive oxygen species and further damage to mtDNA and the maintenance of immunogenic neopeptides, leading to potential immune-mediated damage.

A number of studies have linked mt deletions with cell death and cell death with inflammation, a characteristic of some autoimmune diseases (62, 63). Mirabella et al. (64) found apoptotic changes in the muscle fibers of 36 assayed muscle biopsies from patients with mtDNA deletions and tRNA point mutations. Similar findings in vitro were reported by Asah et al. (65). Because apoptotic cell death is an active, energy-dependent process, defects in mt function render cells more susceptible to apoptosis. Moreover, cell
death has been shown to cause the release of mt Ags that can induce inflammation. A link between inflammatory mt Ags and autoimmunity has been uncovered by a number of researchers (62, 63). Campbell et al. (18) found abundant mt deletions in multiple sclerosis patients. They observed that neurons with high heteroplasmy and mtDNA deletions were evident in both young and aged multiple sclerosis patients. Neurodegeneration would be the result of respiratory deficiency in the neurons and/or chronic inflammation caused by the neopeptides encoded by mutated DNAs (mtDNA, pathogens' DNA). An increased mutational burden in autoimmune rheumatoid arthritis patients further adds support to the concept that neopeptides from mutated mtDNA are implicated in the pathogenesis of inflammatory autoimmunity (17, 66, 67).

Most studies to date implicating mitochondria in inflammation and autoimmunity have focused on the role of innate responses of monocytes/macrophages and neutrophils to the mt components released during cell death (62, 63, 67). The data presented in the present study provide evidence that the adaptive immune system, namely CD8 + and CD4 + T cells, respond to mtDNA-derived neopeptides. Mitochondria, unlike microbial pathogens, are present within the host cells, and the mutations in mtDNA can result in

FIGURE 6. Characterization of native phenotype of T cells recognizing neopeptide P9. (A) Representative flow cytometry plot of two independent experiments. Each experimental condition was done in duplicate in each of the two experiments. Numbers in the gated area of the dot plots correspond to percentages. (B) FMO control was used to determine background levels of tetramer staining. PBMCs were thawed and rested for 2 h at a density of $2 \times 10^6$ cells/well in a round-bottom microtiter plate. Cells were first stained with tetramer followed by staining for surface markers as described in Materials and Methods.

FIGURE 7. CD8+ T cells expanded by self and bacterial homologs of neopeptide P9 peptide bind A*02/P9 tetramers. (A) Representative flow cytometry plot of two independent experiments. Each experimental condition was done in duplicate. Numbers in the gated area of the dot plots correspond to percentages. (B) Summary of CD3+CD8+ A*02/P9 tetramer+ T cells. All data are expressed as means ± SEM. Statistical significance was analyzed relative to no Ag control, but including costimulation (NoAg + CD80) for each of the peptides (P9, self, and bacterial homologs of P9). T cells from donor OA1 were expanded in the presence of self and bacterial homolog peptides, respectively, and subsequently stained with the P9-specific tetramer, as described in costimulation assay (see Materials and Methods) and Fig. 3. Flow cytometric analyses were performed on a lymphocyte-gated population. In all figures, FMO control was used to determine background levels of tetramer staining. *p < 0.05, **p < 0.01 by unpaired t test.
the persistence of immunogenic peptides and thus contribute to the recognition of mtDNA-derived neopeptides by effector T cells. This immune surveillance could be preventing the pathological accumulation of cells with defective mitochondria but may also contribute to the onset or exacerbation of autoimmunity in susceptible individuals due to immunological cross-reactivity between mt neopeptides and self-homologs.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


