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TITLE: **Identification of Novel Myelin-Associated CD4+ T cell Autoantigens Targeted in MS Using a High-Throughput Gene Synthesis Technology**

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14. ABSTRACT
Myelin-specific CD4+ T cells are implicated in the pathogenesis of MS. Attention has focused on the autoantigens MBP, MOG, PLP, and MOBP however, it is unclear whether these are the major targets of the CD4+ T cell response in MS. Over 300 myelin-associated proteins have been identified in the CNS, most of which have not been evaluated as antigens in MS. The purpose of this project was to test a high throughput gene synthesis and T cell screening approach to identify new MS autoantigens. We built a synthetic minigene library encoding the peptide compliment of 342 myelin-associated proteins fused to the LC3 targeting sequence that delivers peptides into the class II antigen presentation pathway. Library accuracy was determined by sequencing and specific amplification of accurate minigenes for a subset of 50 myelin proteins. Minigenes were pooled in a 96 well plate format, transcribed *in vitro,* and mRNA pools were transfected into activated B cells as antigen presenting cells in T cell stimulation assays. The library was validated using an influenza hemagglutinin-specific CD4+ T cell clone. To identify novel class II myelin epitopes, we screened the library using peripheral CD4+ T cells from a flaring MS patient. T cells that responded to library encoded peptides by upregulating CD154 expression were enriched and expanded as T cell lines for further screening to identify the specific peptide targets. We conclude that CD4+ T cell antigens can be detected using minigene encoded peptides but that an enrichment step is required to increase sensitivity

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Table of Contents

INTRODUCTION

CD4+ T cells specific for myelin-associated autoantigens are implicated in the pathogenesis and progression of multiple sclerosis (MS). To date however, attention has focused on responses against an extremely small number of autoantigens including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and myelin associated oligodendrocyte basic protein (MOBP), due primarily to the abundance of these proteins in the CNS, their ability to elicit MS-like disease in the mouse experimental autoimmune encephalitis model, and the presence of T cells reactive to these antigens in the peripheral blood of MS patients (1,2). However, despite these criteria, it remains unclear whether these autoantigens represent disease initiating antigens or are in fact the major targets of the CD4+ T cell response in MS.

Recent proteomic studies of myelin preparations using new protein isolation procedures have revealed that myelin consists of a surprisingly large set of proteins (> 300) (3). Moreover, the detection of autoantibody and T cell responses against Contactin-2/TAG-1 in MS patients strongly suggests that additional antigens remain to be identified (4). Technologies enabling large-scale, unbiased screens to comprehensively identify novel autoantigens associated with myelin sheath destruction target a gap in the knowledge of MS etiology and disease progression.

The purpose of this one year Concept Award was to test a novel, high throughput gene synthesis and T cell screening technology in order to comprehensively screen myelin proteins with the goal of identifying new CD4+ T cell autoantigens targeted in MS. The objectives of this study were to 1) build a synthetic minigene library encoding the complete peptide compliment of approximately 300 myelin-associated proteins fused in frame with the LC3 autophagosomal targeting sequence that delivers peptides into the HLA class II pathway; 2) to validate the library using CD4+ T cell clones specific for known antigens; and 3) to test the library in a single screen of CD4+ T cells from a MS subject with active disease to determine if novel epitopes are identified.

BODY

Task 1: Build a synthetic minigene library encoding the complete peptide compliment of approximately 300 myelin-associated proteins fused in frame with the LC3 autophagosomal targeting sequence that delivers peptides into the HLA class II pathway.

1. *Evaluation of class II targeting sequences for minigene construction:*

Prior to the start of this award we performed an evaluation of known HLA class II targeting sequences to determine which sequence produced the most robust targeting of in-frame peptides to the intracellular pathway for class II antigen presentation. For these experiments minigenes encoding a known influenza epitope, hemagglutinin (HA) 306-318 presented by DRB1*0401, were fused in-frame with one of three class II targeting tags: KFERQ, LC3, or LAMP. As a control, no class II targeting sequence was added to the HA epitope. Delivery of the minigene encoded HA peptide into the class II pathway was tested by transfecting minigene transcripts into DRB1*0401 B cells that served as antigen presenting cells (APC), followed by recognition of the HA epitope by a CD4 T cell clone specific for HA 306-318 using IFNγ ELISPOT as the readout. In this preliminary study, both the LC3 and LAMP fusion proteins stimulated IFNγ production above the levels observed in cells transfected with the HA epitope with no targeting sequence, showing that minigenes utilizing these tags successfully

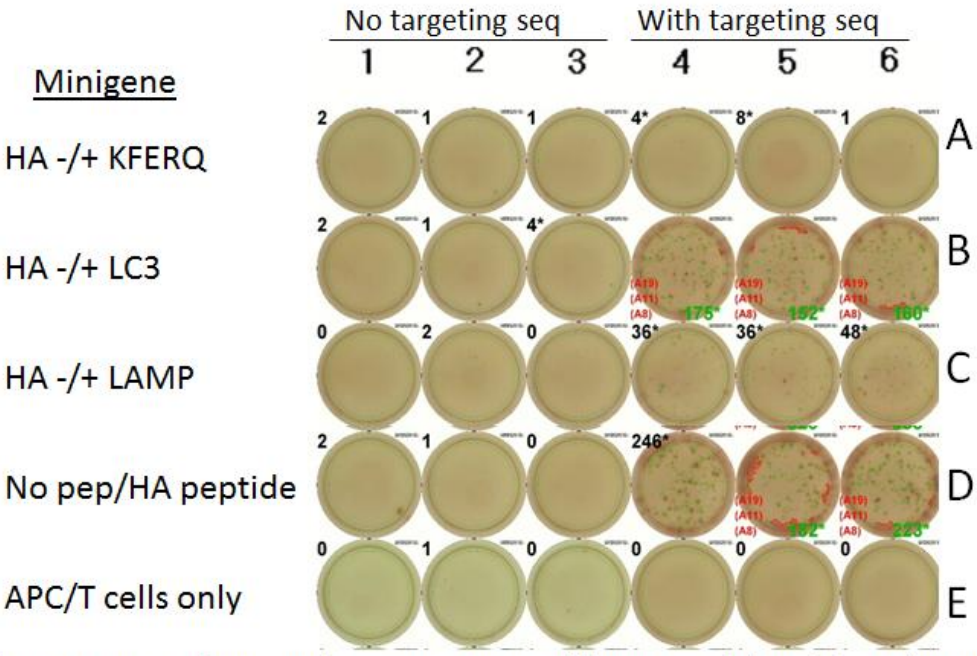

Figure 1. LC3 autophagosomal sequence targets minigene encoded peptides to the HLA class II presentation pathway. Synthetic minigenes were constructed containing the nucleotide sequence encoding the influenza HA 306-318 peptide fused to the indicated targeting sequence. DRB1*0401 B cells expanded with CD40L and IL-21 were Amaxa transfected with in vitro transcripts from the minigenes and then incubated with a T cell clone specific for HA 306-318 in the context of DRB1*0401. T cell activation was detected by IFN-γ ELISPOT. B cells pulsed with HA peptide served as a positive control.

delivered a known epitope into the HLA class II pathway (Figure 1). We chose the LC3 sequence for library construction.

2. Library design and construction:

Protein coding sequences for the 342 myelin-associated proteins described by Jahn et al. (3) were downloaded and subdivided into sequential, overlapping 33 amino acid fragments (99 nucleotides each) using standard bioinformatics techniques. Synthetic primer sequences were appended on both ends of the overlapping nucleotide sequences to allow for subsequent PCR amplification of minigene sequences. The final nucleotide length of the overlapping fragments was 142 bp per peptide.

A library of 6,704 synthetic 142 base oligos representing the entire peptidome of the myelin-associated proteins was commercially synthesized by CustomArrays Incorporated. Upon delivery, the oligos were amplified into pools comprising 20 minigenes per pool using primers homologous to the added primer sequences. The 5' primer sequence also included a T7 promoter for subsequent *in vitro* transcription of minigenes. This was followed by an error correction step. This step was necessary because previous work in the Stone laboratory had established that long oligo pools synthesized in this manner had a high error rate (80% of each oligo species had one or more errors). We utilized an error correction strategy known as "Dialout" that involves tagging of the entire Custom Array minigene library with random 16-mers coupled to Illumina sequencing primers. The library was then subjected to Illumina sequencing and accurate minigenes were identified based on homology to the original synthetic library design. Accurate minigenes (N=960) for a 50 protein subset of the library were then chosen using the unique tagging oligonucleotide pairs as primers to amplify individual minigenes. Due to cost considerations, error correction was not performed on the entire myelin library, although it can be applied to the remainder of the library in future studies. After error correction, the sublibrary was repooled, purified and appended to the LC3 coding sequence, a human beta-globin UTR sequence and a 125 base poly A sequence using overlap extension PCR. These new pools were then transcribed into

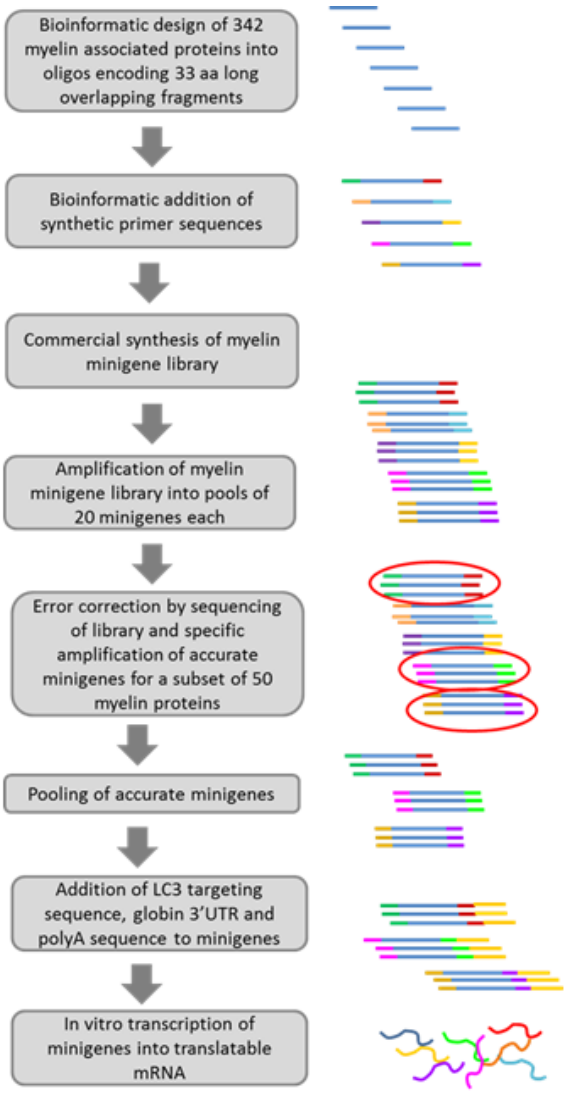

Figure 2. Flowchart of myelin minigene library construction.

translatable mRNA using *in-vitro* transcription. A flow diagram of the library construction is shown in Figure 2.

Task 2: Validate the library using CD4+ T cell clones specific for known antigens.

1. *Validation using T cell clones of known antigen specificity:*

We originally proposed to validate the myelin minigene library using existing DRB1*1501 CD4+ T cell clones specific for MOG and MOBP that had been expanded from class II tetramer-positive cells from a MS patients. However, when these clones were expanded from frozen stocks we were unable to obtain reproducible responses to MOG and MOBP peptides *in vitro*. Therefore, we took an alternative approach that utilized a DRB1*0401 CD4 T cell clone specific for HA 306-318 that demonstrated a robust response to peptide antigen *in vitro*. We acknowledge that this represents a foreign antigen response while we will be detecting autoantigen responses that are likely to have lower avidity; however the HA-specific T cell clone did allow us to validate the functionality of our minigene library. A minigene encoding the HA306-318 nucleotide sequence was cloned in frame with the LC3 targeting sequence as described above, *in vitro* transcribed, and mRNA was then transfected into DRB1*0401 B cells that had been expanded *in vitro* using CD40 ligand (CD40L) expressing L cells and IL-21 using Amaxa transfection. The transfected B cells (2×10^5/well) were used as APC for the HA-specific T cell clone (1×10^3/well) in a T cell stimulation assay using IFN-γ ELISPOT as readout. As shown in Figure 1, we detected a robust response to the HA 306-318 minigene, comparable to the HA peptide positive control, indicating that the minigene library design was capable of delivering peptides to the HLA class II pathway and that antigen-specific CD4+ T cells can be detected using expanded B cells transfected with minigene translatable mRNAs as APC.

2. *Validation using peripheral CD4+ T cells:*

In a separate project, the Stone Lab evaluated detection of peripheral blood CD4+ T cell responses to foreign antigens using the LC3 minigene library design. Minigenes encoding HLA class II epitopes from Epstein-Barr virus (EBV), Cytomegalovirus, influenza and tetanus toxoid linked to the LC3 tag were constructed and *in vitro* transcribed mRNA was transfected into autologous B cells expanded from healthy control subjects as described above. Unmanipulated CD4+ T cells isolated directly *ex vivo* were challenged with autologous, transfected B cells as APC and T cell stimulation was detected using IFN-γ ELISPOT assays. These experiments established that the signal to noise ratio from unmanipulated peripheral CD4+ T cells was insufficient for unambiguous detection of responses against common HLA class II target antigens, i.e. the sensitivity of the assay was not sufficient to detect antigen-specific CD4+ T cells.

To circumvent this problem, we added an enrichment step to enrich and expand CD4+ T cells responding to a particular antigen prior to detection. For antigen specific enrichment we utilized upregulation of CD154 upon exposure to cognate antigen-HLA class II which has been well-documented as a readout for rare antigen activated T cells (5,6). We took advantage of technology developed at our institution to enrich $CD154^+$ T cells using magnetic bead isolation

for enrichment of antigen specific cells directly from peripheral blood (unpublished). As a validation of this approach and our myelin library, we enriched myelin-reactive peripheral CD4+ T cells from a control subject that had previously been shown to have myelin reactive CD4+ T cells in peripheral blood. Banked PBMC from this subject was used to isolate total CD4+ T cells by negative selection and the T cells ($2x10^6$/well) were then exposed to autologous expanded B cells (10^6/well) that were transfected with minigenes encoding MBP, MOG, and MOBP, or an irrelevant minigene. After a 3h incubation in the presence of anti-CD40 to prevent CD154 downregulation, T cells that had upregulated CD154 expression were stained with PE-conjugated anti-CD154 and anti-PE magnetic beads, followed by magnetic column enrichment. $CD154^{hi}$ cells were subsequently purified by flow sorting (Figure 3). We found that this enrichment strategy resulted in isolation of twice as many myelin responsive $CD154^{hi}$ cells as an irrelevant antigen. Following sorting, CD154 positive T cells were expanded using non-specific stimuli to increase the number of antigen responsive T cells for a subsequent detection step with the MBP, MOG, and MOBP minigenes using ELISPOT. These experiments are currently underway.

Task 3: Test the myelin minigene library in a single screen of CD4+ T cells from a MS subject with active disease.

To determine if the high throughput minigene library approach could identify novel myelin antigens targeted in MS, we utilized PBMC from a therapy-naïve MS patient experiencing a disease flare. Since this patient was newly diagnosed at the time of blood draw, it wasn't possible to classify the disease as relapsing remitting or progressive.

For library screening, total B cells were isolated and expanded using CD40L expressing L cells and IL-21. Error-corrected minigenes (N=960) representing a subset 50 myelin proteins were *in vitro* transcribed and pooled into a total of eight pools. The pooled mRNAs were transfected into autologous expanded B cells (10^6/well), and incubated overnight to allow minigene expression. Total CD4+ T cells were isolated by negative selection from banked PBMC and rested overnight to bring CD154 levels to baseline. CD4+ T cells ($2x10^6$/well) were then exposed to transfected B cells for 3h in the presence of anti-CD40 to prevent CD154 internalization. $CD154^+$ T cells were enriched by magnetic bead isolation as described above, and $CD154^{hi}$ and

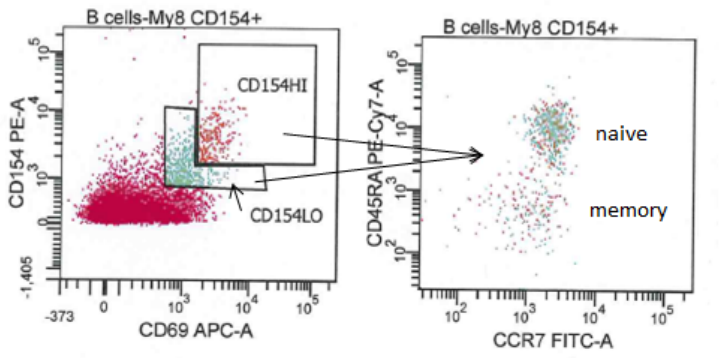

Figure 3. CD4+ T cells upregulate CD154 in response to B cells expressing myelin minigenes. Peripheral CD4+ T cells ($2x10^5$ per well) were rested overnight to bring CD154 expression to baseline. Autologous expanded B cells (10^6 per well) were transfected with minigene mRNAs encoding myelin associated proteins and incubated for 8h to allow gene expression. T cells were added to B cells and incubated for 3h in the presence of anti-CD40 to allow CD154 upregulation. CD154+ cells were enriched by magnetic bead isolation followed by flow cytometry. T cells that were antigen stimulated were identified as CD154+/CD69+. $CD154^{hi}$ cells were a mix of naïve and memory T cells.

$CD154^{lo}$ cells were isolated by flow sorting. >100 $CD154^{hi}$ cells were isolated for each of the eight myelin pools. These T cells are currently being expanded using non-specific stimuli prior to rescreening with the myelin minigene sublibrary to validate specificity and identify target peptide specificity by pool deconvolution.

KEY RESEARCH ACCOMPLISHMENTS

- Built a synthetic library of 6,704 minigenes encoding overlapping peptides of 342 myelin-associated proteins fused in frame with the LC3 autophagosomal targeting sequence.
- Performed error correction on a subset of 960 minigenes encoding 50 unique myelin associated proteins
- Validated that the LC3 minigenes were processed and presented as class II antigens using an antigen specific CD4+ T cell clone.
- Developed a strategy to enrich antigen specific T cells from peripheral blood using CD154 upregulation and magnetic bead isolation coupled with flow cytometry.
- Screened peripheral blood CD4+ T cells from a flaring MS patient versus the error corrected myelin minigene library and isolated CD154 positive cells which are being expanded to identify target peptide specificity.

REPORTABLE OUTCOMES

- Development of a synthetic library of 6,704 minigenes encoding overlapping peptides of 342 myelin-associated proteins, including a sub-library consisting of 960 error-corrected minigenes encoding 50 myelin proteins.

CONCLUSIONS

We developed a synthetic library of 6,704 minigenes encoding overlapping peptides of 342 myelin-associated proteins. Minigene nucleotide sequences were fused in frame to the LC3 autophagosomal targeting sequence to enable trafficking to the HLA class II antigen presentation pathway. Error correction was performed on a subset of 960 minigenes, encoding 50 unique myelin proteins. We validated the functionality of the library by successful stimulation of a CD4+ T cell clone specific for influenza HA 306-318. However, in experiments performed for another study, we were unable to detect CD4+ T cell responses to minigenes encoding class II epitopes from EBV, CMV, influenza, and tetanus toxoid using unmanipulated T cells from peripheral blood. To circumvent this sensitivity problem, we developed a strategy to enrich CD4+ T cells responding to minigene encoded peptides using CD154 upregulation, magnetic bead enrichment, and flow cytometry. Following flow cytometry, enriched cells were expanded using non-specific stimuli to increase the number of antigen responsive T cells for subsequent rescreening with the stimulating minigenes in order to validate specificity and identify target peptide specificity. To identify novel myelin antigens targeted in MS, we screened the error-corrected myelin minigene library with peripheral blood CD4+ T cells from a flaring MS subject prior to initiation of therapy. Utilizing eight pools of myelin minigenes, we isolated over 100 $CD154^{hi}$ CD4+ T cells/pool. These T cells are currently being expanded for rescreening. We conclude that the minigene approach can target peptides to the HLA class II antigen presentation pathway, resulting in CD4+T cell stimulation. However, the sensitivity of detection of rare antigen-specific CD4+ T cells in peripheral blood presents a hurdle which we have attempted to overcome using CD154 enrichment of antigen responsive T cells. Analysis of the expanded T cell lines resulting from our library screening will reveal whether this is a successful approach or if it should be used in combination with isolation of particular T cell subsets such as Th1 or Th17 to improve sensitivity.

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