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Predicting the effects of potentially therapeutic modified peptides on polyclonal T cell populations in a mouse model of multiple sclerosis

Evan L Sauer*, Elisabeth Trifilieff[†], Judith M Greer*[‡]

*The University of Queensland, UQ Centre for Clinical Research, Brisbane, Australia

[†]Laboratoire d'Imagerie et de Neurosciences Cognitives (LINC), Université de Strasbourg/CNRS, France

[‡]Corresponding Author:

Dr Judith M Greer, UQ Centre for Clinical Research, Bldg 71/918, Royal Brisbane & Women's Hospital, Brisbane QLD 4029, Australia. Tel: +61 7 3346 6018. E-mail: j.greer@uq.edu.au

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Abbreviations: APL, altered peptide ligand; LNC, lymph node cell

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Abstract

Altered peptide ligands (APLs) have routinely been studied in clonal populations of Th cells that express a single T cell receptor (TCR), but results generated in this manner poorly predict the effects of APLs on polyclonal Th cells *in vivo*, contributing to the failure of phase II clinical trials of APLs in autoimmune diseases such as multiple sclerosis (MS). We have used a panel of APLs derived from an encephalitogenic epitope of myelin proteolipid protein to investigate the relationship between antigen cross-reactivity in a polyclonal environment, encephalitogenicity, and the capacity of an APL to provide protection against experimental autoimmune encephalomyelitis (EAE) in SJL mice. In general, polyclonal Th cell lines specific for encephalitogenic APLs cross-reacted with other encephalitogenic APLs, but not with non-encephalitogenic APLs, and vice versa. This, alongside analysis of TCR Vβ usage, suggested that encephalitogenic and non-encephalitogenic subgroups of APLs expand largely non-cross-reactive Th cell populations. As an exception to the rule, one non-encephalitogenic APL, L188, induced proliferation in polyclonal $CD4^+$ T cells specific for the native encephalitogen, with minimal induction of cytokine production. Co-immunization of L188 alongside the native encephalitogen slightly enhanced disease development. In contrast, another APL, A188, which induced IL-10 production without proliferation in $CD4^+$ T cells specific for the native encephalitogen, was able to protect against development of EAE in a dose-dependent fashion when co-immunized alongside the native encephalitogen. These results suggest that testing against polyclonal Th cell lines *in vitro* may be an effective strategy for distinguishing between potentially therapeutic and non-therapeutic APLs.

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1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS) characterised by the formation of lesions both in CNS white matter tracts and in CNS grey matter (McDonald et al., 2001; Polman et al., 2005; Polman et al., 2011). These sites of inflammation are believed to be initiated by adaptive immune responses directed against antigens derived from CNS myelin, although innate responses play a major role in the evolution of the lesion. Current treatments for MS invariably act without discriminating between pathogenic autoimmune responses and homeostatic immune function, either by inhibiting inflammatory responses, depleting certain classes of immune cells, sequestering immune cells within lymph nodes, or by limiting the access of immune cells to the CNS, with associated risks and side-effects (Lim and Constantinescu, 2010; Benkert et al., 2012; Steinman, 2012). Therapies that are able to selectively disrupt immune responses directed against autoantigens may be able to prevent the development of lesions at an earlier stage, thereby proving safer and less intrusive than treatments which suppress autoimmunity by disrupting systemic immune function.

Altered peptide ligands (APLs), created by introducing amino acid substitutions within a peptide at positions that interact with the TCR, can have the capacity to actively antagonize T cell activation in an antigen-specific manner. Because of this property, APLs have been studied as therapeutic agents in animal models of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE). However, attempts to translate the successes seen in these models to the treatment of human diseases have failed in clinical trials (Bielekova et al., 2000; Kappos et al., 2000). There are a number of contributing factors behind these failures (reviewed in Sauer et al., 2015); one, in particular, has been the way in which candidate APLs

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have been selected for use. Typically, APLs have been studied on the basis of their ability to antagonize monoclonal T cell clones *in vitro* (Kuchroo et al., 1994; Vergelli et al., 1997; Anderton et al., 1998), on the apparent assumption that the APLs would function in a similar manner to suppress autoreactive T cells *in vivo*. While the homogeneity of monoclonal T cell lines can simplify the interpretation of data, they poorly represent the characteristics of physiological, polyclonal T cell immune responses. Variations between TCRs involved in a polyclonal response crease a spectrum of affinites for an antigen and variation in the antigenic residues that contribute to TCR ligation. Sampling many monoclonal T cell responses (as in Vergelli et al., 1997) does not guarantee the construction of a representative picture, as it does not account for the paracrine effects of cytokine production on T cell differentiation and activation. T cell anergy and antagonism, particularly, can be over-ridden by exogenous IL-2, which is produced by activated T cells (Dure and Macian, 2009).

Furthermore, APLs are often immunogenic in their own right, expanding populations of APL-reactive T cells with the potential to cross-react with the native antigen (Nicholson et al., 1995; Das et al., 1997; Nicholson et al., 1997; Anderton et al., 1998; Anderton et al., 1999; Ausubel et al., 1999). This effect cannot be detected using monoclonal autoantigen-specific T cells. APL-reactive T cells will produce cytokines which may mediate bystander suppression of autoimmune responses (Nicholson et al., 1995; Greer et al., 1997; Nicholson et al., 1997; Cloake et al., 2014) or which may exacerbate inflammatory responses (Anderton et al., 1998; Anderton et al., 1999). Often, therefore, the immunogenic properties of an APL, rather than its direct effect on autoreactive T cells, have proven more valuable in the treatment of disease.

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We sought to investigate how the immunogenic and immunomodulatory properties of an APL affect its inherent encephalitogenicity and its capacity to provide protection against the induction of EAE, in a polyclonal T cell environment, using a relapsing-remitting model of EAE in SJL mice. In this model, disease can be actively induced by an epitope of myelin proteolipid protein (PLP) spanning residues 178-191, which contains phenylalanine at position 188 ($PLP_{178-191}$; F188) (Greer et al., 1992). An APL created by introduction of alanine at this position (A188) has been found to show only a low level of cross-reactivity with F188-specific T cells, but to also expand a population of A188-responsive T cells that produce Th2 cytokines and suppress EAE, at least in part through bystander suppression (Greer et al., 1997; Cloake et al., 2014).

In the present study, we have expanded upon these findings using a panel of eight APLs carrying conservative and non-conservative amino acid substitutions at position 188. We show that in a polyclonal setting, APLs which activate polyclonal F188-reactive Th cells either induce disease directly or exacerbate F188-induced disease, while APLs which activated polyclonal A188-reactive Th cells do not. Different APLs preferentially activate T cells bearing specific T cell receptor Vβ chains.

These results suggest that testing against polyclonal Th cell lines *in vitro* may be an effective strategy for distinguishing between potentially therapeutic and non-therapeutic APLs.

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2. Materials & Methods

2.1. Peptides

A set of eight peptides including native murine $PLP_{178-191}$ (F188) and seven APLs containing substitutions of alanine (A), aspartic acid (D), glycine (G), leucine (L), serine (S), tryptophan (W), and tyrosine (Y) in place of F at position 188 were synthesized. Each peptide was named according to the amino acids at position 188 (A188, D188, G188, F188, L188, S188, W188 and Y188). The properties of the different residues at position 188 are shown in Table I. The peptides were reconstituted to 5 mg/mL in 0.2 M acetic acid and stored at -20 °C. For experimental use, the stock solutions were diluted in PBS (for *in vivo* testing) or in tissue culture medium, consisting of RPMI-1640 (Sigma-Aldrich, St Louis MO, USA) supplemented with 10% heat-inactivated Serum Supreme, 20 mM HEPES, 2 mM L-glutamine (all from Lonza, Basel, Switzerland), and 50 mM 2-mercaptoethanol (Sigma-Aldrich).

2.2. Induction of EAE

Female SJL/J mice between six and eight weeks of age were immunised s/c on the flanks with 100 μg of peptide emulsified in complete Freund's adjuvant (CFA) (Difco) containing 4 mg/mL heat-killed *M. tuberculosis* H37RA (Difco). At the same time, and again after three days, each mouse received 300 ng pertussis toxin (List Biological Laboratories, Inc., CA, USA) in PBS i/v via the tail vein. Mice were monitored daily from day seven until euthanasia at day forty, recording weight and grading the clinical severity of EAE according to a standard scale (0 = normal, 1 = weak tail, 2 = limp tail, 3 = hind limb weakness, 4 = hind limb paralysis, 5 = moribund) (Cloake et al., 2014).

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2.3.Generation and maintenance of antigen-specific T cell lines

Female SJL/J mice between six and eight weeks of age were primed by immunization with a total 100 μg of peptide in the same manner as for the induction of EAE. After ten days, the draining (axilliary and inguinal) lymph nodes were removed into tissue culture medium and teased apart to give a lymph node cells (LNC) suspension. Polyclonal T cell lines (TCLs) were generated from LNCs by regular rounds of stimulation with peptide. Stimulated TCLs were maintained in long-term culture at approximately 1×10^6 cells/mL in flat-bottom 24-well plates at 37°C in a 5% CO_2 in air environment. Each T cell line was stimulated at least three times with peptide and irradiated (3000 rad) syngeneic spleen cells as antigen-presenting cells (APCs), allowing at least four weeks in tissue culture medium supplemented with 20 U/mL recombinant human IL-2 (Hoffman-LaRoche Inc., Basel, Switzerland) between each round of stimulation, to ensure specificity prior to experimental use.

2.4. Proliferation assay

Antigen-specific TCLs were suspended in tissue culture medium and plated in triplicate into 96-well round-bottomed plates (Corning, Inc., Corning NY, USA) at 3×10^4 cells per well. Each well was stimulated by addition of antigen at specified concentrations (or, for unstimulated controls, tissue culture medium alone), together with 3×10^5 irradiated syngeneic spleen cells as APCs. Plates were cultured for 48 hours, after which time 0.5 μCi of ^{3}H-thymidine (ICN Pharmaceuticals, Montreal, Canada) was added to each well for a further 18 hours. ^{3}H-thymidine incorporation was measured using a liquid scintillation counter. The proliferation induced by each peptide was expressed as a stimulation index (SI), determined by the formula: SI = mean cpm of antigen-containing triplicate wells / mean cpm of control triplicate wells.

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2.5. Flow cytometric analysis of Vβ usage

T cells were stained with FITC-TCR Vβ and PE-CD4 antibodies as described, then analysed by flow cytometry using a two-laser four-colour BD FACScalibur flow cytometer and BD CellQuest Pro version 0.3 software. Lymphocytes were gated according to forward- and side-scatter characteristics, then on expression of CD4. Statistics were collected from a histogram plot of FITC-TCR Vβ intensity within the gated population.

In addition, proliferation of specific Vβ expressing T cell populations in response to antigenic stimulation was determined using T cells labelled with 5 μM Cell Trace Violet (CTV, Life Technologies, Carlsbad CA, USA), an analog of carboxyfluorescein diacetate succinimidyl ester (CFSE). The CTV-labelled cells were stimulated as described earlier. After 72 hours, cells were washed three times in sterile PBS-azide wash solution (PBS, 1% serum, 0.5% sodium azide), then resuspended in PBS-azide wash solution containing FITC-conjugated monoclonal antibodies against mouse TCR Vβ subunits (FITC-TCR Vβ; Becton Dickinson, Franklin Lakes NJ, USA), PE-conjugated monoclonal antibodies against mouse CD4 (PE-CD4; Becton Dickinson), or isotype controls. Staining proceeded for 30 minutes at 4 ºC, then unbound antibodies were removed by three washes in PBS-azide wash solution at 4 ºC. All samples were resuspended in 500 μL PBS-azide wash solution, then analysed by flow cytometry using a three-laser, ten-color Beckman Coulter Gallios flow cytometer and software. Lymphocytes were gated according to forward- and side-scatter characteristics, then on expression of CD4. Quadrant statistics were collected from a scatter plot comparing FITC-TCR Vβ to CTV intensity within the gated population. The gating strategy is shown in Supplementary Figure 1.

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2.6. Measurement of cytokine production using Cytometric Bead Arrays

Supernatants were collected from each T cell line three days after stimulation and stored at -80°C. Production of IL-2, IL-10, IL-17A, IFN-γ, and TNF-α was measured using a Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson) according to the manufacturer's instructions. Measurements of fluorescence were collected using a two-laser four-colour BD FACScalibur flow cytometer and BD CellQuest Pro version 0.3 software, and analysed using BD FCAP Array v3 software.

2.7. Preparation of total RNA

A188- and F188-specific TCLs were stimulated as described. After six hours, total RNA was extracted using a QIAGEN RNeasy kit (QIAGEN, Venlo, Netherlands), according to the manufacturer's instructions. The total RNA sample was stored at -80 °C prior to use. Total RNA concentration and purity was estimated by spectrophotometry, measuring absorbance at wavelengths of 260 nm (A260) and 280 nm (A280).

2.8. *Real-time polymerase chain reaction*

Quantitative RT-PCR was performed using the RT2 Profiler PCR Array system (QIAGEN). Complementary DNA was prepared from total RNA. The RT-PCR reaction was performed using RT2 SYBR Green Mastermix (QIAGEN), which contained HotStart DNA Taq Polymerase, and customized 96-well arrays pre-loaded with optimized quantities of gene-specific primers, as well as controls for genomic DNA, reverse-transcription, and positive PCR. The array was sealed and run, using a Bio-Rad iCycler and iQ5 Optical System

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Software v1.0.410, for one cycle at 95 °C for 10 minutes, then for forty cycles at 95 °C for 15 seconds, 60 °C for 1 minute. Fluorescence data was collected during the 60 °C incubation and used to automatically calculate the threshold cycles (CT) for each well of the array. After the RT-PCR was completed, dissociation (melting) curve analysis was performed to verify PCR specificity by incubation at 95 °C for 1 minute, 65 °C for 2 minutes, then from 65 °C to 95 °C at 2 °C/minute, during which time fluorescence data was collected. Results were validated and analyzed using PCR Array Data Analysis Software from QIAGEN.

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.01. Disease incidence was compared between control and test groups using Fisher's Exact test. For other comparisons, unless otherwise indicated, data were first tested to see if they fitted a normal distribution, and then statistical comparisons were made using a one-way analysis of variance (ANOVA) method for either parametric or non-parametric data, with Bonferroni's correction for multiple comparisons. Data was deemed significant at $p < 0.05$.

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3. Results

3.1. Encephalitogenic potential of APLs

We first tested the ability of the native encephalitogenic peptide, F188 (NTWTTCQSIA**F**PSK), and the seven other APLs in our panel to induce EAE in SJL mice. The APLs contained amino acid substitutions at position 188 which differed with respect to size, charge, and acidity, and included aromatic and aliphatic groups. Animals immunized with peptides containing aromatic residues at position 188 (F188, W188 and Y188) developed EAE (Table II). The incidence of disease was lower in mice immunized with W188 (50%) and Y188 (75%) compared to those immunized with F188 (100%), and while the maximum clinical score in animals which developed clinical disease was no different between these three groups of animals, the duration of the acute phase of disease was significantly shorter in mice immunized with W188 or Y188, compared to those immunized with F188. No animals which received A188, G188, L188, S188 or D188 lost any weight or developed clinical signs of disease within 40 days of immunization, nor were any signs of inflammation or demyelination present in spinal cords or brains of these mice upon histological analyses (data not shown).

3.2. Cross-reactivity between polyclonal T cells specific for each APL

To explore the relationship between encephalitogenicity and cross-reactivity, we tested the recall responses of short term T cell lines generated from LNC of mice that had been primed by immunization with each peptide and then cultured *in vitro* for 3 rounds of stimulation with the immunizing peptide (20 μg/ml for the first round of stimulation, 10 μg/ml for the second round of stimulation, and 5 μg/ml for the third stimulation). Three to four weeks after the final stimulation (once T cells had returned to a resting state), T cell lines were tested for their

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cross-reactivity with the panel of eight peptides, by measuring proliferative responses through the incorporation of ^{3}H-thymidine. The maximal proliferative response to each peptide across the range of concentrations tested is summarized in Figure 1, and the responses of each line to each peptide across the range of concentrations are shown in Supplementary Table 1.

Three main patterns of reactivity were observed. Firstly, T cell lines generated from LNCs of animals immunized with G188 responded strongly only to G188, with limited cross-reactivity to any other peptide. Secondly, T cell lines generated from LNCs of animals immunized with A188, D188, L188 and S188 displayed similar patterns of cross-reactivity, mounting the most vigorous responses to the non-encephalitogenic peptides A188, D188, G188, L188 and S188, but responding to the encephalitogenic peptides F188, W188 and Y188 only at the highest concentrations tested. These results demonstrate that these non-encephalitogenic peptides retained the capacity to induce immune responses *in vivo*. It was also likely, given the high level of cross-reactivity between these populations, that A188, D188, L188 and S188 expanded a common pool of T cells, which were not stimulated by peptides containing an aromatic amino acid at position 188.

Finally, there was a similar pattern of cross-reactivity amongst the T cell lines generated from LNCs of animals immunized with the peptides F188, W188 and Y188. All three of these lines reacted strongly to F188, although W188-specific T cell lines did not proliferate very strongly to Y188, and Y188-specific T cells did not proliferate very strongly to W188. F188-specific T cell lines proliferated strongly to F188, W188 and Y188. Surprisingly, F188-specific T cell lines also mounted a robust proliferative response to L188, evident even at the lowest concentrations of peptide tested. These results suggest that the encephalitogenicity of F188, W188 and Y188 was based on their ability to activate a common pool of autoreactive T

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cells. Non-encephalitogenic peptides, with the exception of L188, failed to activate this population, but expanded an independent T cell pool that could not recognise native F188.

3.3. Vβ usage

The low level of cross-reactivity between A188- and F188-specific T cell lines and F188 and A188 peptides suggested that T cell populations activated by A188 and F188 shared little TCR identity. To begin to explore this possibility, three independently-generated TCLs specific for A188 and three independently-generated TCLs specific for F188 were stained with FITC-conjugated antibodies against TCR Vβ subunits and with PE-conjugated anti-CD4 antibody. SJL mice carry the "a" haplotype of the *Tcrb* gene complex, and express TCR Vβ1, Vβ2, Vβ3, Vβ4, Vβ6, Vβ7, Vβ10, Vβ14, Vβ15, Vβ16, Vβ17a, Vβ18, and Vβ19 (Luckheeram et al., 2012). In these experiments, we used a panel of antibodies specific for TCR Vβ2, Vβ3, Vβ4, Vβ6, Vβ7, Vβ14, and Vβ17a to identify Vβ usage in the TCLs. Antibodies against the other TCR Vβ subunits utilized by SJL/J mice were not commercially available.

There was some variability from one line to another, but in the A188-specific lines, Vβ2 and Vβ3 expressing T cells predominated in all lines (Table III). In contrast, Vβ2 and Vβ3 accounted for only a small fraction of the $CD4^+$ T cell population in the F188-specific lines. Instead, Vβ4 and $Vβ17^a$ positive T cells predominated in the F188-specific $CD4^+$ T cell populations. These data confirm that the immune responses against A188 and F188 are dominated by T cells bearing distinct TCRs, but allow for the possibility that some TCRs may be represented in both A188- and F188-reactive T cell pools.

TCR Vβ4 was also the most common subunit in W188- and Y188-specific T cell lines (contributing 31.3% and 34.1% of TCRs, respectively), suggesting that a common

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encephalitogenic TCR Vβ4^{+} T cell population may be present among F188-, W188- and Y188-reactive T cells. The most abundant Vβ TCR types in L188-specific T cells were TCR Vβ17^{a} and Vβ4.

We tested the cross-reactivity of each TCR Vβ subgroup in each cell line to the APLs A188, F188 and L188, using CTV to measure proliferation (Figure 2). It was apparent that, among the A188-specific T cell line, T cells expressing TCR Vβ4, the subunit most abundant among F188-specific T cells, were also the most cross-reactive with F188. Similarly, F188-specific T cells expressing TCR Vβ17^{a} demonstrated less cross-reactivity to A188 than the broader F188-specific population, which matched the low proportion of TCR Vβ17^{a+} T cells in A188-specific T cell lines. F188 and L188 typically induced proliferation of similar percentages of each Vβ subtype in both lines, with the exception of Vβ4^{+} F188-specific T cells, suggesting that this population of cells may be largely responsible for the encephalitogenic potential of the F188-specific response. These results point to functional heterogeneity within the polyclonal populations, related to TCR expression, but also suggest that some T cell clones expressing identical TCRs might be found among populations expanded by A188, F188 or L188.

3.4. Cytokine production

We measured the levels of cytokines produced by A188- and F188-specific T cells following stimulation with A188, F188 or L188 using a cytometric bead array. Supernatant was collected from A188-specific or F188-specific T cells three days after incubation with irradiated syngeneic splenocytes alone (to provide a background measure of cytokine production by unstimulated cells), or with irradiated syngeneic splenocytes plus 10 μg/mL of either A188, F188 or L188 (Figure 3).

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Stimulation of A188-specific T cells with A188 peptide induced significantly increased production of all cytokines, apart from IL17a. The fold change in IL-10 production was particularly strong, with a greater than 250-fold increase compared to levels in unstimulated A188-specific T cells. Stimulation of A188-specific T cells with F188 or L188 peptides did not induce any cytokine production that was significantly elevated above background levels.

Using F188-specific T cells stimulated under similar conditions with 10 μg/mL of F188, the greatest fold changes in cytokine levels were for IL-17A, TNF-α, IFN-γ, and IL-2. Stimulation of F188-specific T cells with 10 μg/mL of A188 induced increases in IFN-γ and IL-10 production that were of equal or greater magnitude to the increases induced by stimulation with F188. In contrast, levels of IL-17A, TNF-α and IL-2 were significantly decreased in F188-specific T cells stimulated with A188 compared to when they were stimulated with cognate antigen. The levels of cytokines produced by F188-specific T cells following stimulation with 10 μg/mL of L188 were similar to those induced by A188, with the exception of IL-10 and IFN-γ.

3.5. Different genes are activated in A188- and F188-specific T cells following activation

We measured expression of a set of 26 genes related to T cell activation, Th effector subtype-specific transcription factors, and the induction of anergy in A188- and F188-specific T cell lines following activation with either A188, F188 or L188. Resting T cells from A188- and F188-specific lines exhibited similar levels of expression for most genes tested; however, 6 of the 26 tested genes, namely *Gata3*, *Maf*, *Tbx21*, *Rnf128*, *Mapk1*, and *Cdkn1a*, were differentially expressed (Figure 4a). *Maf*, which is associated with Th2 and regulatory T cells, was expressed more strongly in A188-specific T cells than in F188-specific T cells. *Gata3*

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expression was greater in F188-specific T cells than in A188-specific T cells. *Rnf128* (encoding GRAIL) was predominantly expressed in F188-specific T cells, and *Mapk1* (encoding ERK) and *Cdkn1a* (encoding p21/Waf1) were slightly more abundant in A188-specific T cells.

Compared to unstimulated A188- or F188-specific T cells, cells stimulated with their cognate antigen showed similar patterns of gene expression, with significant upregulation of *Tbx21*, *Cd40lg*, *Nfatc1*, *Fasl*, *Ctla4* and *Cdkn1a*, and significantly downregulation of *Foxp3*, *Nfatc2* and *Nfatc3*, *Mapk14*, *Blc2*, *Tob1*, and *Plcg1*: this pattern of gene expression therefore seemed to reflect the signature of activated T cells in our populations (Fig 4b).

In comparison with the cognate antigen, A188-specific T cells stimulated with either F188 or L188 failed to upregulate *Cd40lg*, *Nfatc1*, *Fasl*, or *Ctla4*, and did not significantly downregulate *Nfatc2*, *Blc2*, or *Rnf128* (Fig 4b and Supplementary Table 2). *Tbx21* was significantly upregulated following stimulation with F188 (although not as strongly as after stimulation with A188), but was not significantly upregulated following activation with L188. F188-specific T cells stimulated with either A188 or L188 also failed to upregulate *Cd40lg*, *Ctla4, Nfatc1* and *Fasl*. *Tbx21* was also upregulated and *Nfatc2*, *Blc2*, and *Rnf128* were downregulated in F188-specific T cells by activation with both A188 and L188, although to a lesser degree than by activation with F188.

3.6. Comparison of capacity of A188 and L188 to protect against induction of EAE

Although the L188 peptide was not itself encephalitogenic (Table II), could expand the same common pool of T cells as the protective APL A188 (Figure 1 and Supplementary Table 1), and did not induce high levels of proinflammatory cytokines in F188-specific T cells (Figure 3), the substantial degree of cross-reactivity between encephalitogenic F188-specific T cells

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and L188 peptide (Figure 1) and lack of induction of IL-10 production (Figure 3) raised the question as to whether L188 would act in a protective manner, similar to A188. In a disease setting, where an expanded pool of autoreactive F188-specific T cells is driving CNS inflammation, the capacity of L188 to induce proliferation of F188-primed LNC might mean that L188 would exacerbate the autoimmune response instead of protecting against it. To test this, we examined the effects of A188 and L188 on the course of EAE actively induced by F188.

Co-administration of the A188 APL alongside 100 µg F188 at molar ratios of 0.2:1, 1:1 or 5:1 A188:F188 decreased the incidence of clinical disease in a dose-dependent manner (Table IV & Figure 5). In animals which developed clinical signs of disease, disease severity in mice receiving 1:1 A188:F188 was significantly less than in mice receiving F188 alone (2.0 ± 0.4 versus 3.3 ± 0.2, respectively; $P = 0.01$), but not in mice receiving either 0.2:1 A188:F188 or 5:1 A188:F188. There were no significant difference in the day of onset ($P = 0.40$). All mice treated with A188 recovered completely within 7 days after onset of EAE, and no relapses of disease were seen within the observation period in this group (Figure 5a).

In contrast, all mice co-immunized with L188 APL together with F188 (at molar ratios of 0.2:1, 1:1 or 5:1 L188:F188) developed clinical disease, with elevated maximum clinical scores relative to mice receiving F188 alone (Table 2 and Figure 5a). There were no significant differences in the day of onset for mice receiving any dose of L188 compared to F188-immunized mice (Figure 4b). By day 10 after onset of the first attack of EAE, some of the mice in the F188 alone and L188-treated groups had started to relapse (Figure 5a). Thus, L188 did not provide any prophylactic protection against F188-induced EAE, and appeared to slightly worsen disease.

4. Discussion

A major problem encountered in designing APLs for therapeutic applications is that there is no empirical formula to decide what will constitute a therapeutically useful APL, and trial and error has played a large role in deciding which APLs should be taken forward to animal and clinical trials. Because of the large number of assays that need to be done using such an approach, testing of APLs has usually been carried out using T cell clones. Working with identical T cells simplifies the interpretation of the relationships between different signalling events and functional effects. But even among monoclonal populations, the differentiation of isolated naïve T cells into specific effector cell subtypes is stochastic, and it is autocrine and paracrine signalling between T cells, and between T cells and APCs, that directs Th cell differentiation (Nakayama and Yamashita, 2010). If the cross-talk between cells is influential in determining the differentiation of monoclonal T cell populations, then it is likely to play an even greater role in shaping and coordinating the response of polyclonal T cell populations to an antigen *in vitro*, and in determining the form of an adaptive immune response to antigen *in vivo*. It could therefore be expected that effects of APLs on clonal T cell populations might not bear a strong relationship to their effects in live animals and in the human patients, and this has indeed been found to be the case. APLs that successfully suppress the development of model autoimmune diseases have often done so by unforeseen mechanisms. Some APLs that were predicted to suppress autoimmunity, as they could antagonise the activation of select autoreactive T cell clones *in vitro*, instead proved to exacerbate disease. Furthermore, the amelioration or exacerbation of autoimmune disease by an APL has often been the

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consequence of the APL's immunogenic properties, rather than its direct effects on autoreactive T cells.

In the current study, we have investigated the dependence of encephalitogenic and protective immune responses on the amino acid occupying position 188 of the immunodominant epitope $PLP_{178\text{-}191}$. T cell lines specific for non-encephalitogenic peptides proliferated strongly to other non-encephalitogenic peptides, suggesting that each of these peptides were recognized by T cells bearing a common pool of TCRs. These T cell lines did not proliferate strongly in response to F188, indicating limited capacity to recognize native $PLP_{178\text{-}191}$. Similarly, T cell lines specific for F188 proliferated strongly to the two other encephalitogenic APLs (W188 and Y188), but not (as a general rule) to non-encephalitogenic APLs. These results suggest that the capacity of these peptides to induce EAE in SJL mice was closely related to the populations of T cells that they expanded.

The exception to the rule was the reactivity of F188-specific T cells to L188. L188 was non-encephalitogenic and expanded A188-specific T cells, but it also induced strong proliferation among T cell lines specific for F188. Evidently, L188 acted as an agonist for both sets of T cells. Because of this, it was not clear whether L188 would, like A188, provide protection against EAE induced by F188, or whether it would exacerbate disease. We confirmed that L188 was not encephalitogenic in SJL mice when administered without F188. However, unlike A188, co-administration of L188 alongside F188 provided no protection against induction of EAE; instead, disease was exacerbated. L188 behaved as a weak or partial agonist towards F188-specific T cells, inducing proliferation and strong downregulation of *Rnf128* and *Bcl2*, but poor cytokine production; indeed, levels of cytokines produced by F188-specific T cells following stimulation with L188 were no higher than those produced

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following stimulation with A188, which was only able to induce a very low level of proliferation in F188-specific T cells.

Consistent with data obtained from proliferation assays, cell populations expanded by A188 and F188 were dominated by T cells carrying distinct TCR Vβ subunits. Among F188-specific lines, most T cells carried TCR Vβ4 or Vβ17a, while among A188-specific lines, most carried TCR Vβ2 or Vβ3. Furthermore, TCR $V\beta4^+$ T cells from both A188- and F188-specific lines proliferated most strongly in response to the encephalitogenic peptide F188. Interestingly, Vβ4 has also been shown to be the predominant TCR type used by SJL/J mice in the response to an encephalitogenic peptide of myelin basic protein (MBP_{92-103}) (Padula et al., 1991; Kalman et al., 1994).

Applying both anti-TCR Vβ antibodies and the fluorescent marker CTV to distinguish between the proliferative responses of T cell subpopulations demonstrated functional heretogeneity within our polyclonal populations, related to TCR expression, and suggested that T cell clones expressing identical TCRs might be found among populations expanded by A188 or F188. Anti-TCR Vβ antibodies are compatible with *in vitro* functional experiments, though they are imprecise in identifying specific TCRs, and do not currently span the diversity of TCR Vβ subunits expressed in mice. Spectratyping the TCRs borne by T cell clones from each population lacks such compatibility, but would confirm whether particular TCRs are common to some or all groups.

Parallels can be drawn between the effects of L188 on the induction of EAE and the difficulties encountered in other EAE models and in clinical trials of APLs in MS. For example, APLs of a MBP epitope, which were expected to behave as antagonists *in vivo*,

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based on their effects on monoclonal T cells *in vitro*, instead exacerbated EAE in mice by agonizing autoreactive polyclonal T cells (Anderton et al., 1998). A similar phenomenon was encountered in a phase II clinical trial in which the APL increased the frequency of myelin-specific Th1 cells in peripheral circulation in MS patients (Bielekova et al., 2000). These results, and our own results, demonstrate that the capacity of APLs to act as agonists *in vivo* can facilitate both amelioration and exacerbation of autoimmune disease. Therefore, when considering the therapeutic potential of an APL, it is necessary to consider both its effects on autoreactive T cells and its own immunological properties under physiological conditions.

This is all the more important as the agonistic effects of APLs *in vivo* have often been responsible for providing protection against disease by expanding populations of T cells that play a regulatory role through the production of anti-inflammatory cytokines. This is evident in numerous $CD4^+$ T cell-mediated models of autoimmune diseases. For example, in experimental autoimmune myasthenia gravis (EAMG), an APL of the acetylcholine receptor (AchR)-α subunit acted as an antigen-specific inhibitor of polyclonal T cell responses *in vitro* and *in vivo* and ameliorated the clinical manifestations of established EAMG (Katz-Levy et al., 1997). Because the *in vivo* response to native antigen could be inhibited by the transfer of splenocytes from APL-treated animals, it is likely that amelioration of disease was due to the activity of a regulatory T cell population (Paas-Rozner et al., 2001). Indeed, immunization with the APL increased the production of IL-10 and TGF-β (Faber-Elmann et al., 2000; Paas-Rozner et al., 2001), increased the proportion of $CD4^+CD25^+$ cells (Paas-Rozner et al., 2003; Aruna et al., 2005; Ben-David et al., 2005; Aruna et al., 2006; Ben-David et al., 2007), and increased Forkhead box p3 (Foxp3) expression (Aruna et al., 2005; Ben-David et al., 2007). Likewise, an APL of collagen II (CII), effective against progression of collagen-induced arthritis (CIA) in rats (Zhoa et al., 2008; Li et al., 2009) was associated with the induction of

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Th2 cells and reduced levels of IFN-γ (Li et al., 2009), and reduced titres of Th1-associated IgG2a (Zhoa et al., 2008). Also in CIA, an APL based on influenza virus hemagglutinin 308-317 (which is structurally similar to the dominant CII epitope, with higher affinity for DR1/DR4 (Dessen et al., 1997)) had similar effects on the course of disease in DBA/1 mice, through induction of $FoxP3^+$ Treg cells and associated suppression of IFNγ and IL17 production (Sun et al., 2012).

Our results illustrate the need to consider both the immunogenic and immunomodulatory properties of an APL in tandem, in order to identify or exclude APLs as potential therapeutic agents in the treatment of autoimmune diseases. An experimental strategy that examines the effects of APLs on the broader Th cell repertoire then identifies principle sub-populations and characterizes their distinctive responses to APLs while preserving a polyclonal context may provide a more realistic picture of how APLs modulate immune responses. An approach such as this may resolve the impasse that has arisen between the complexity of physiological immune responses and the clarity of observing monoclonal T cell populations.

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Tables

Table I. Properties of peptides used in this study.

Designation	**Sidechain structure at residue 188**	**Properties**
A188	$-CH_3$	nonpolar, aliphatic
D188	$-CH_2-C(=O)O^-$	acidic
F188	$-CH_2-C_6H_5$	nonpolar, aromatic
G188	-H	nonpolar, aliphatic
L188	$-CH_2-CH_2-CH(CH_3)_2$	nonpolar, aliphatic
S188	$-CH_2-OH$	polar
W188	$-CH_2-$ indole (N-H)	nonpolar, aromatic
Y188	$-CH_2-C_6H_4-OH$	polar, aromatic

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Table II. Encephalitogenicity of peptides containing substitutions at position 188 of $PLP_{178-191}$ in SJL/J mice. Mice were immunised with 100 µg of each peptide using a standard protocol for active induction of EAE, and followed for 40 days.

Group	n	Incidence of Acute Disease	Maximum Clinical Score (mean ± sem) §	Day of onset (mean ± sem) §	Duration in days (mean ± sem)
A188	4	0%	0	n.a.	n.a.
D188	4	0%	0	n.a.	n.a.
F188	5	100%	3.2 ± 0.2	10.4 ± 0.2	8.4 ± 0.7
G188	4	0%	0	n.a.	n.a.
L188	4	0%	0	n.a.	n.a.
S188	4	0%	0	n.a.	n.a.
W188	4	50%	2.8 ± 0.8	10.5 ± 0.5	5.0 ± 2.0
Y188	4	75%	3.2 ± 0.6	13.0 ± 0.0	6.0 ± 0.0

§The maximum clinical score and day of onset are derived only from mice that developed clinical disease (mean ± sem).

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Table III. Percent usage of different Vβ TCR elements by A188- and F188-specific T cell lines.

Vβ type	**A188 T cell line**	**F188 T cell line**
Vβ2	15.6 ± 5.5%*	5.8 ± 0.7%
Vβ3	19.5 ± 5.8%***	2.7 ± 0.6%
Vβ4	8.9 ± 1.7%	37.2 ± 4.5%****
Vβ6	0.6 ± 0.3%	2.4 ± 0.3%
Vβ7	2.5 ± 0.4%	1.6 ± 0.4%
Vβ14	2.8 ± 0.5%	3.2 ± 1.2%
Vβ17a	3.2 ± 0.9%	12.7 ± 2.6%
Remainder	46.9 ± 4.3%*	34.6 ± 0.7%

Results for A188- and F188-specific T cell lines were compared using Bonferroni's multiple comparison test. * $P<0.05$; ***$P<0.001$; ****$P<0.0001$

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Table IV. Effects of A188 and L188 on the induction of EAE.

Group	n	Incidence of Acute Disease	Maximum Clinical Score§	Day of Onset§
F188	13	92%	3.3 ± 0.2	12.3 ± 1.2
L188	4	0%**	n.a.	n.a.
0.2:1 A188:F188	8	63%	3.1 ± 0.4	13.2 ± 1.4
1:1 A188:F188	8	50%*	2.0 ± 0.4*	14.8 ± 1.7
5:1 A188:F188	8	38%*	3.0 ± 0.8	13.3 ± 1.2
0.2:1 L188:F188	4	100%	3.9 ± 0.1	11.0 ± 1.1
1:1 L188:F188	4	100%	4.0 ± 0.4	11.5 ± 0.3
5:1 L188:F188	4	100%	3.8 ± 0.5	12.0 ± 0.4

§The maximum clinical score and day of onset are derived only from mice that developed clinical disease (mean ± sem). **P*<0.05 compared to F188 group; ***P*<0.01.

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Figure Legends

Figure 1. Summary of cross-reactivity of APL-specific T cell lines. Each panel represents the cognate antigen (indicated in upper corners). Each bar represents the maximal level of ^{3}H-thymidine incorporation induced by the indicated peptide, irrespective of peptide concentration, expressed as a proportion of the maximal response to the cognate antigen (black bars). The hatched bar indicates the aberrant response of F188-specific T cells to L188.

Figure 2. Vβ usage of A188- or F188-specific T cells proliferating in response to stimulation by A188, F188 or L188. Each pie graph shows the proportion of cells of each Vβ type that proliferated in response to antigen, out of the total number of cells that were proliferating.

Figure 3. Fold change in cytokine production by A188-specific and F188-specific T cell lines (TCL) following stimulation with A188, F188 or L188. Significance relative to unstimulated T cells (no peptide) is indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Figure 4. Expression of selected genes related to T cell activation, Th effector subtype-specific transcription factors and the induction of anergy in A188- and F188-specific T cell lines. A. Volcano plot comparing gene expression in unstimulated cells. B. Changes in gene expression (compared to unstimulated cells) when A188- and F188-specific T cell lines were activated by either A188, F188, or L188.

Figure 5. Effects of A188 and L188 on the course of EAE. A. Mean clinical score of all animals. B. Disease incidence. Statistical significance at $p < 0.05$ and $p < 0.01$ levels, relative to mice immunised with F188 alone (red), is indicated by one or two symbols, respectively; *

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for 0.2:1 A188:F188 (blue), † for 1:1 A188:F188 (green), ‡ for 5:1 A188:F188 (purple). The log-rank test was used to test significance for B. Error bars represent sem.

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Figure 1

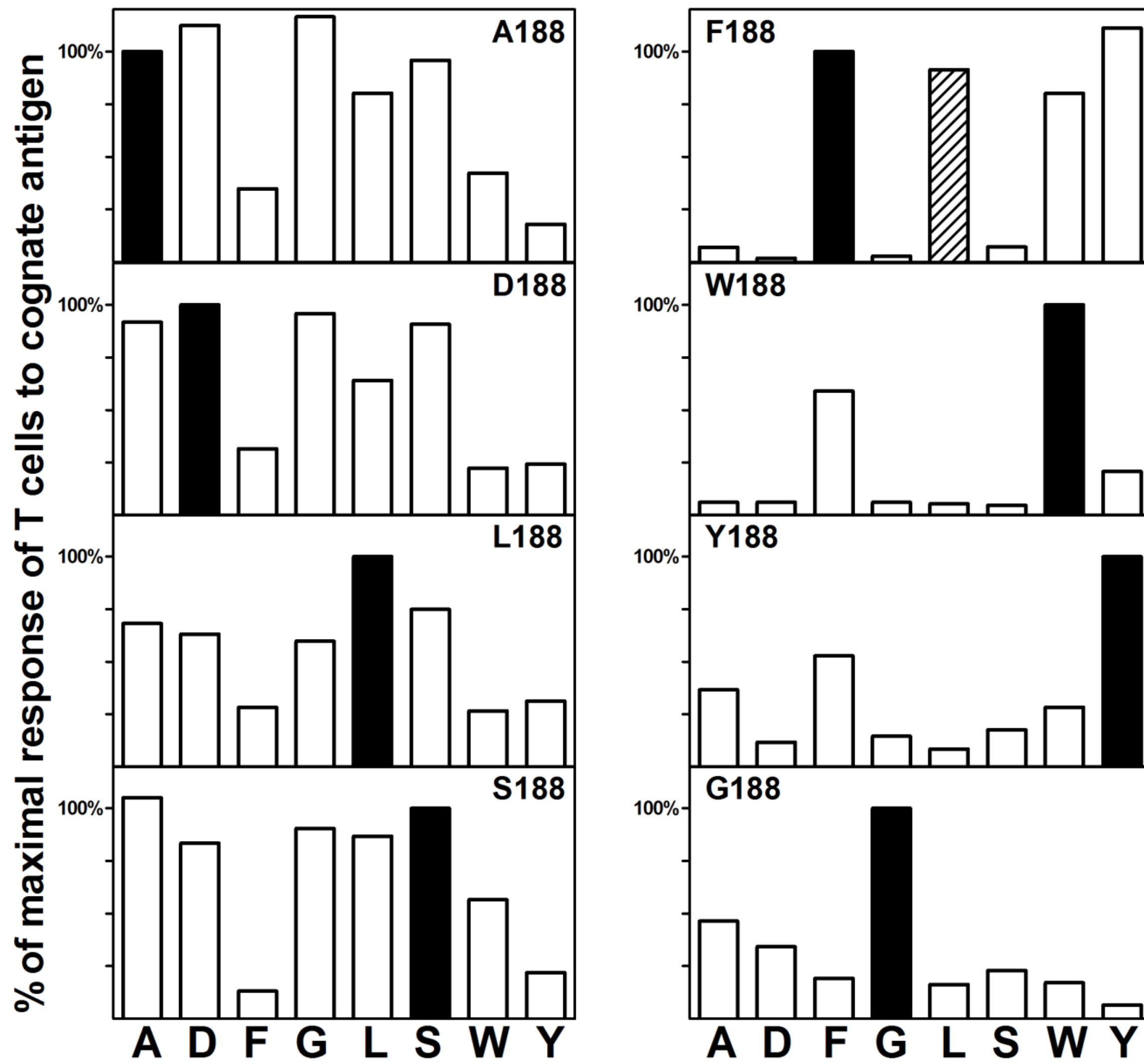

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Figure 2

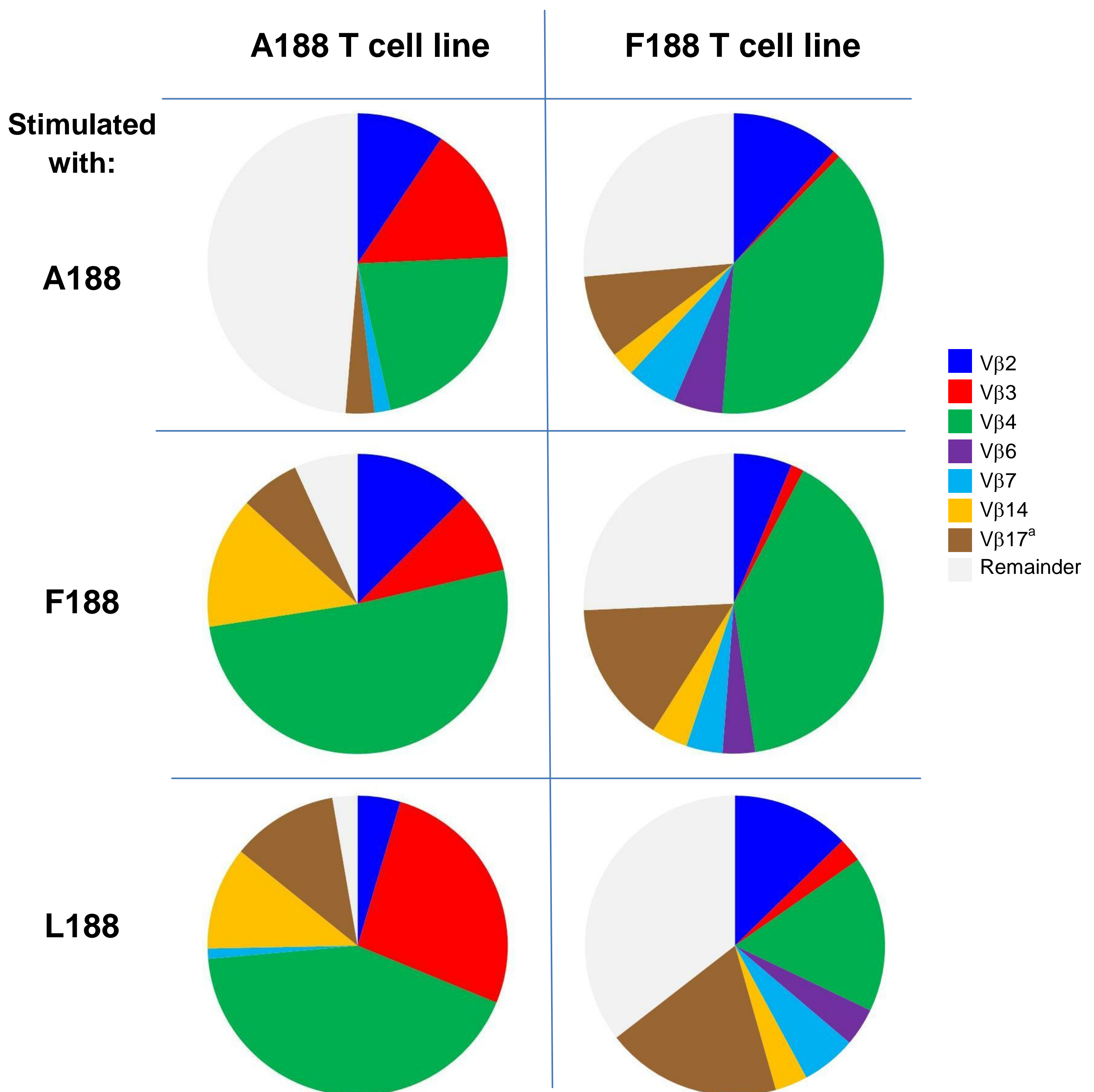

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Figure 3

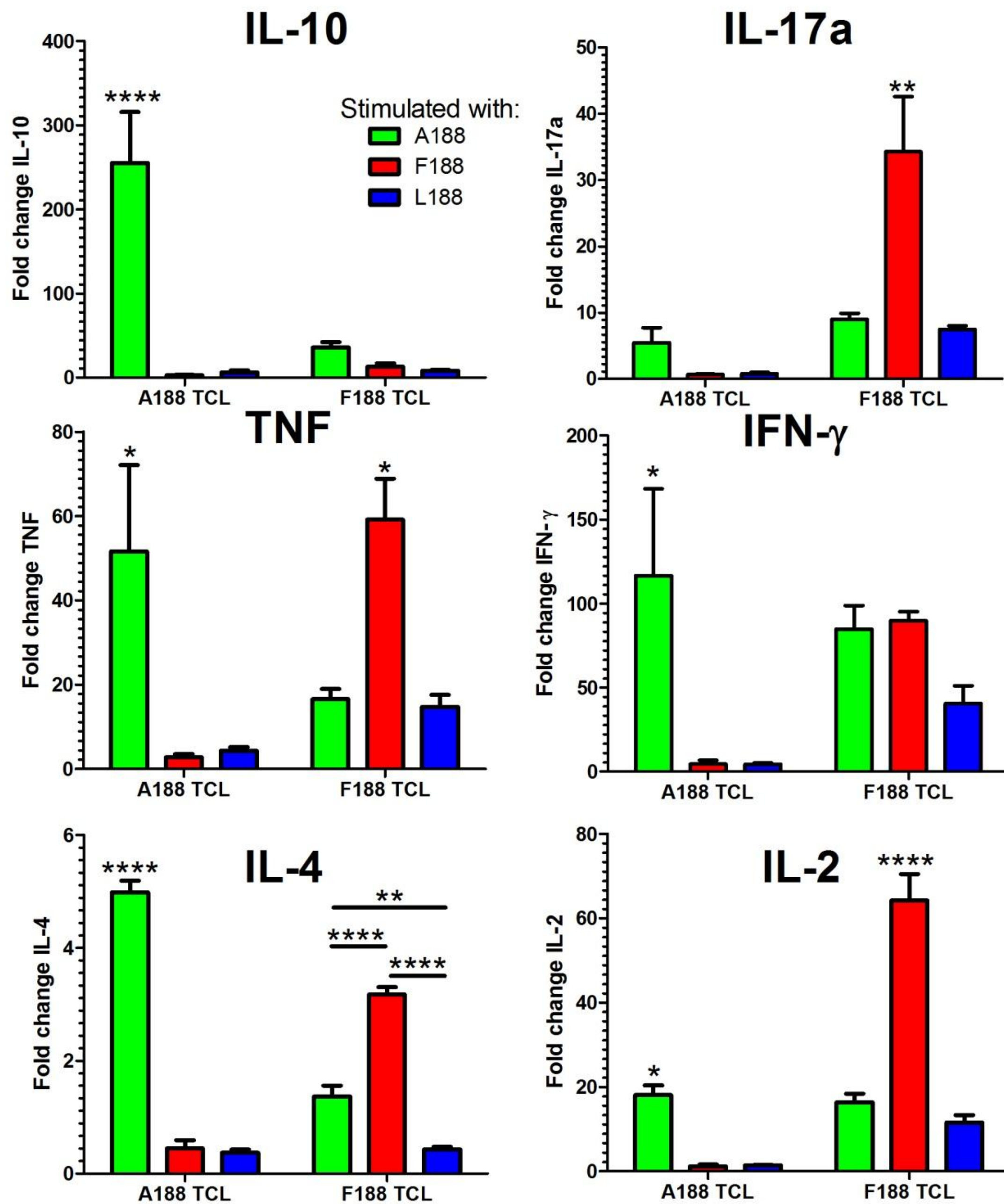

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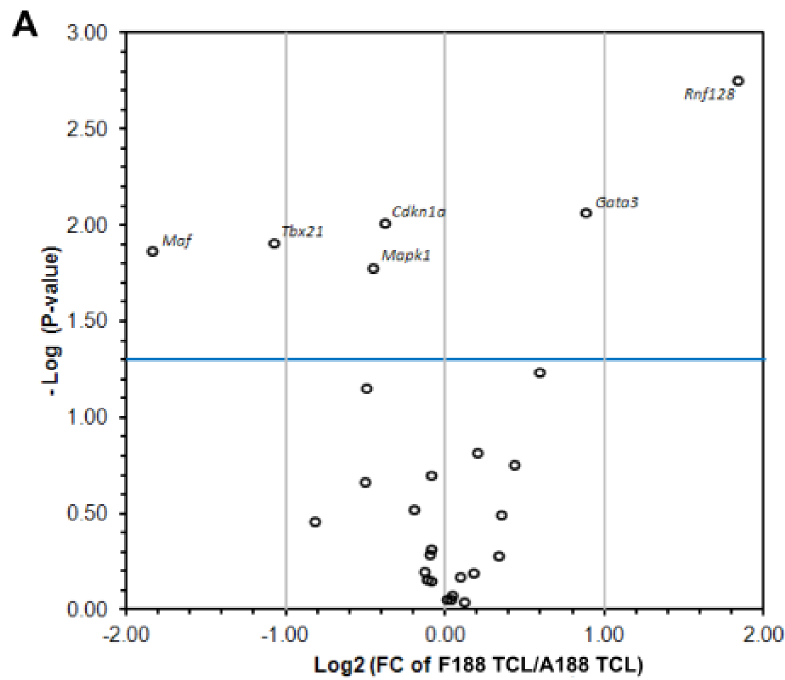
Figure 4



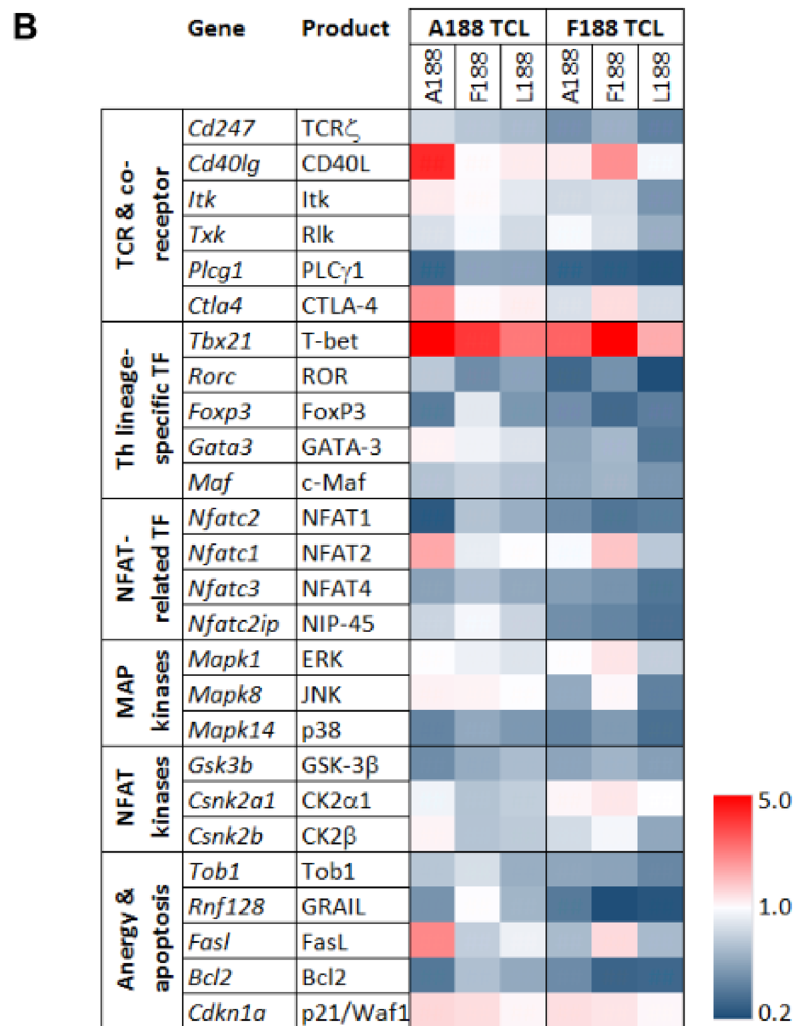


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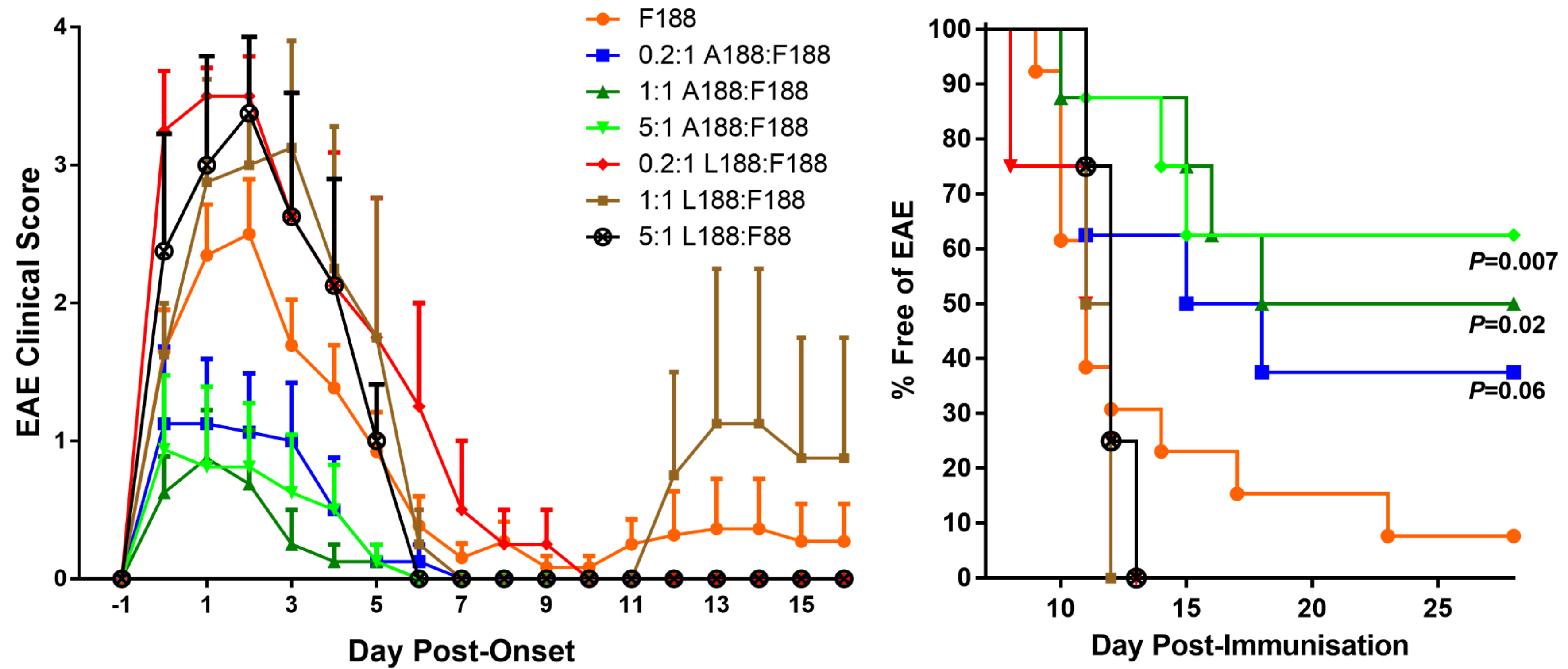
Figure 5.



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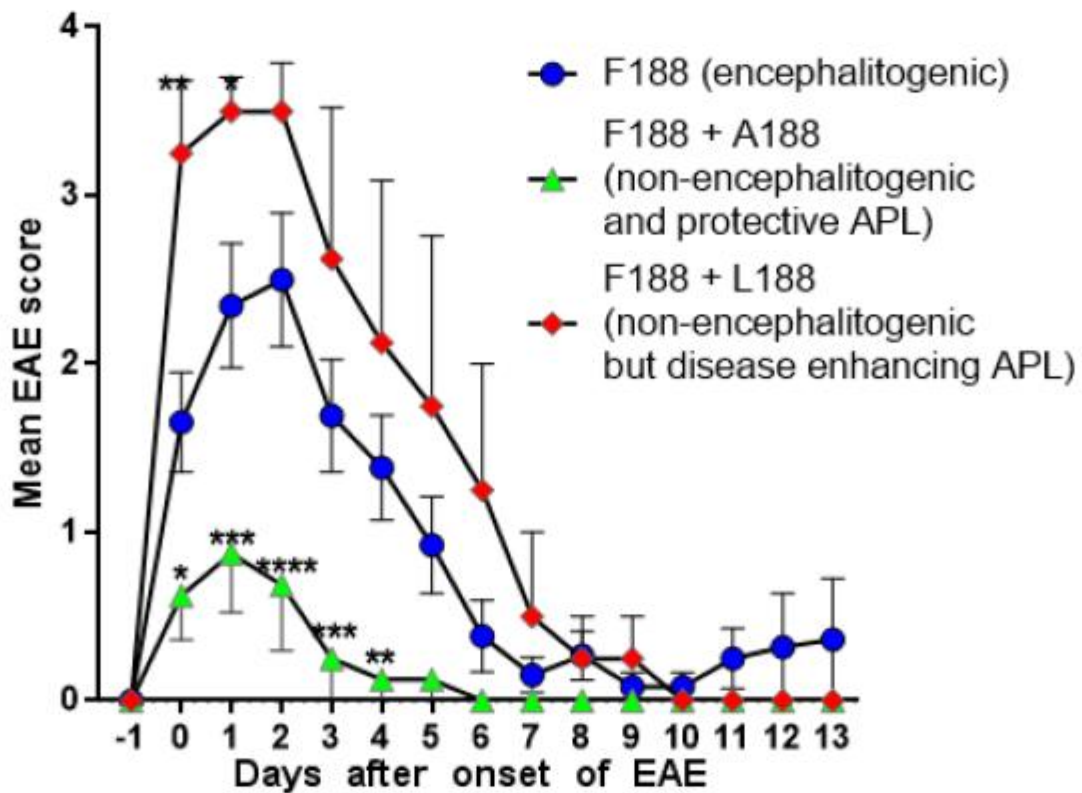
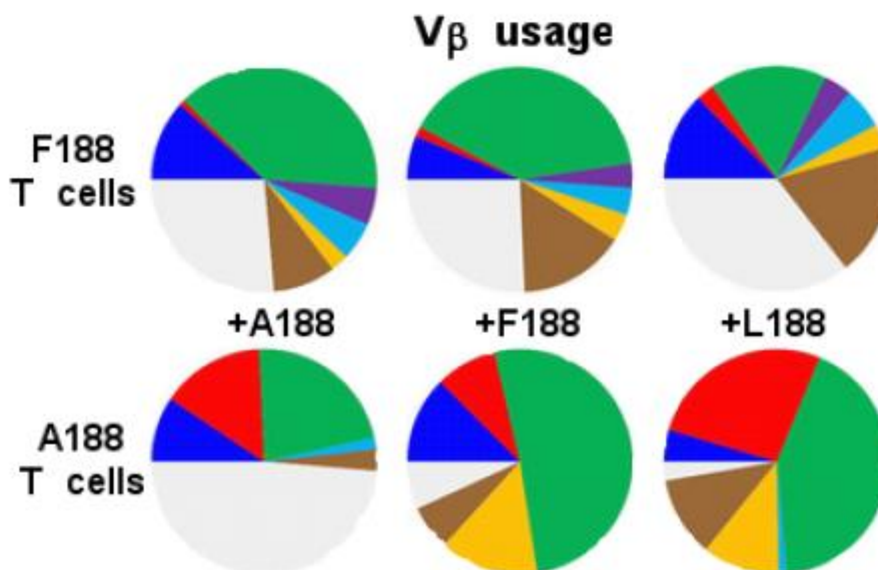
Graphical Abstract





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Highlights

- Effects of APL on polyclonal immune responses were investigated
- Encephalitogenic and non-encephalitogenic APL expand different T cell populations
- Some non-encephalitogenic APL can still enhance encephalitogenicity
- Induction of IL-10 production is a good indicator of a protective APL

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