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Review

PM1-Alpha ELISA: The assay of choice for the detection of anti-PM/Scl autoantibodies?

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ABSTRACT

A characteristic serological feature of patients suffering from the overlap polymyositis and scleroderma (PM/Scl) syndrome are antibodies to the human counterpart of the yeast exosome referred to as the PM/Scl complex. Historically, the detection of anti-PM/Scl antibodies was laborious and relied largely on indirect immunofluorescence and immunodiffusion techniques. In 1992 the major autoantigen PM/Scl-100 was identified and cloned. Subsequently, the major epitopes were mapped and one of these, termed PM1-Alpha, became the antigen for a novel ELISA exhibiting high sensitivity and specificity for the detection of anti-PM/Scl antibodies. Comparative studies with other methods using other PM/Scl autoantigens have shown that the PM1-Alpha ELISA has higher sensitivity and specificity than assays that employed recombinant PM/Scl-75c and PM/Scl-100. Anti-PM1-Alpha antibodies were identified in 55.0% of sera from PM/Scl overlap syndrome patients, but were also seen in 7.9% of SSc and in 7.5% of PM patients. The frequency in other systemic autoimmune diseases and in infectious diseases was significant lower. In summary, the data derived from individual studies suggest that PM1-Alpha may become the “gold standard” for the detection of anti-PM/Scl antibodies.

Contents

1. Introduction

Anti-nucleolar antibodies (ANoA), a subset of anti-nuclear antibodies (ANA), are directed against a number of nucleolar antigens, which include the PM/Scl complex, an antigen target that has been historically associated with a polymyositis (PM), scleroderma (Scl, or systemic sclerosis; SSc) overlap syndrome [1]. The PM/Scl autoantigen was initially described in 1977 in sera of PM patients and some years later the antigen was named the ‘PM/Scl antigen’ when two groups reported that these autoantibodies (aab) were most prevalent in PM/Scl patients [1–4]. In the 1990s, the major antigens PM/Scl-75 and PM/Scl-100 were cloned and the PM/Scl antigen system was identified as the human counterpart of the yeast exosome, a

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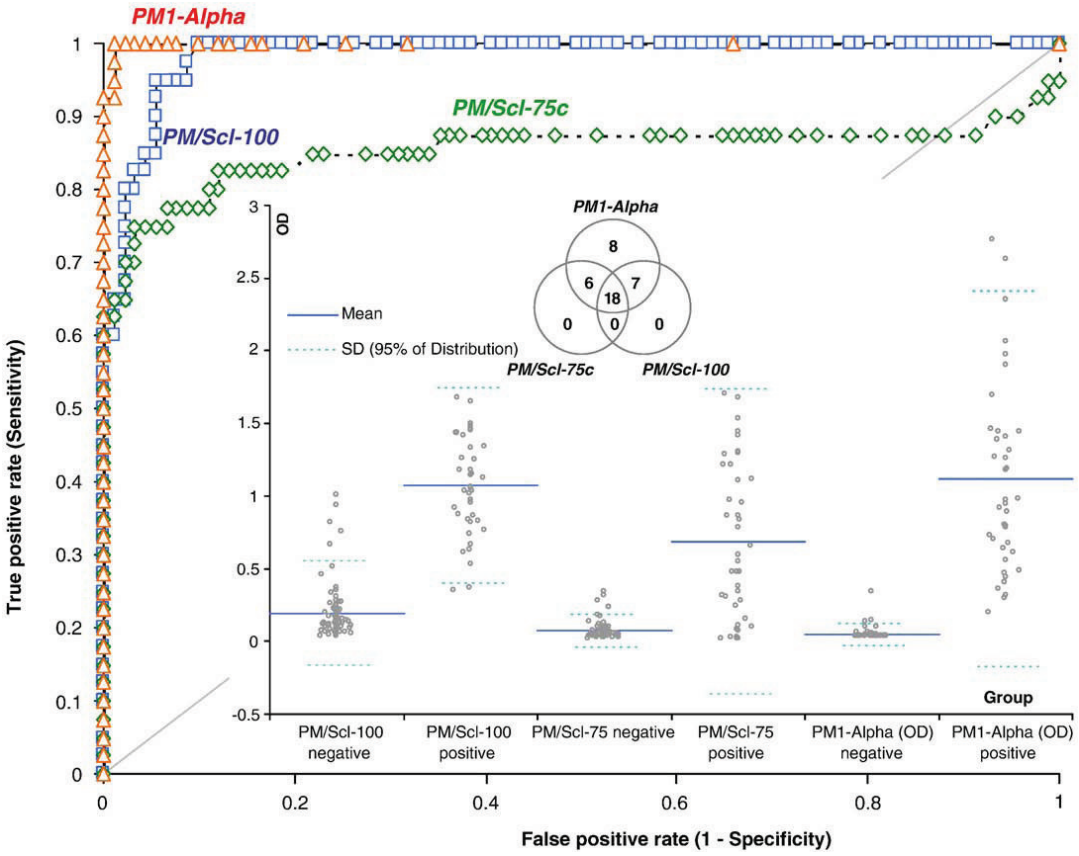

Fig. 1. Reactivity to the major PM/Scl autoantigens PM/Scl-75c, PM/Scl-100, PM1-Alpha. 40 samples with predefined anti-PM/Scl reactivity as defined by indirect immunofluorescence and confirmation methods and 40 controls were tested by ELISA. Receiver operating (ROC) characteristic and comparative descriptive analysis show better discrimination between PM/Scl positive and negative samples using the PM1-Alpha ELISA compared to ELISAs with recombinant PM/Scl-75c or PM/Scl-100. At cut-off values that results in 100% specificity for all three ELISAs 18 samples were positive by all three methods, 7 by PM1-Alpha and PM/Scl-100 and 6 by PM1-Alpha and PM/Scl-75c. Eight samples demonstrated exclusive reactivity to PM1-Alpha. None of the samples negative for anti-PM1-Alpha were positive for antibodies to one or both of the recombinant antigens. [33]. Mean values and standard deviation (SD) of 95% distribution are shown for each group.

macromolecular complex involved in RNA degradation and processing [4–7]. This discovery allowed the purification of the complex and enabled the identification of other subunits [8]. Although not as often recognized by patient sera as PM/Scl-75 and PM/Scl-100, some of these other exosome subunit proteins also proved to be target autoantigens [1].

The ring shaped complex of nine core exosome proteins has been localized to the cytoplasm and nucleoplasm, but is most abundant in the nucleolus [9,10]. The most clinically relevant protein is believed to be PM/Scl-100, which is stably associated with a fraction of the core exosome and also has ribonuclease activity [1].

A variety of techniques have been used to detect anti-PM/Scl antibodies in CTD including the identification of a characteristic ANoA staining pattern as detected by indirect immunofluorescence (IIF) on HEp-2 cells followed by confirmation using double immunodiffusion (ID), immunoprecipitation (IP), immunoblotting (IB) with extractable nuclear antigens, or enzyme linked immunosorbent assay (ELISA) [1] employing purified native or recombinant proteins. The detection of anti-PM/Scl antibodies by IB and IIF, however, is difficult, due to weak reactivity on IB and potential confusion with a number of other ANoA (e.g. anti-fibrillarin, anti-RNA polymerase I, B23/nucleophosphmin) in IIF screening tests. The majority of the anti-PM/Scl ELISA tests use recombinant PM/Scl-100 protein expressed in *E. coli* or in insect cells, because this is the best known autoantigenic component of the PM/Scl complex. Although the reactivity of several other PM/Scl components were tested, aab against these proteins were most commonly found in patients who were also positive for PM/Scl-100 [1,11]. In 2005, however, a new isoform of the PM/Scl-75 protein, termed PM/Scl-75c was shown to have a slightly higher sensitivity than PM/Scl-100 when used in ELISA. The aab reactivity of many other components associated with the exosome has yet to be tested. Combined or multiplexed testing for these and other exosome related proteins might further increase the sensitivity of protein based ELISA assays for anti-PM/Scl reactivity in the future.

Anti-PM/Scl antibodies were primarily found in patients with PM, SSc or DM, with the highest occurrence in overlap syndromes of SSc with PM or DM (polymyositis or dermatomyositis respectively; both referred to as PM/Scl here). A meta-analysis of all studies that used ID, IIF and/or IP to determine anti-PM/Scl reactivity shows that this aab reactivity is found in 31% of all PM/Scl patients, compared to 8% of patients with PM alone, 11% of DM patients and 2% of SSc patients (data not shown). Due to the low prevalence of anti-PM/Scl aab in CTD, the relationship to clinical features is derived from a relatively small number of patients, although multiple studies have addressed the issue in various cohorts of patients and, as described above, anti-PM/Scl aab have

been associated with the PM/Scl overlap syndrome. When combining all these studies, 59% (80/139) of all anti-PM/Scl positive patients are diagnosed with an overlap syndrome. The majority of the remaining patients in all studies had either SSc, PM or DM, or in rare cases inclusion body myositis [12], Sjögren syndrome [13], systemic lupus erythematosus (SLE) [1,14–16], and even acquired haemophilia [17]. The most frequently observed symptoms are muscle weakness and inflammation (myositis), Raynaud's phenomenon (RP) and arthritis [18]. Other reported clinical manifestations include swallowing problems (dysphagia), lung or kidney involvement and mechanic's hands. The PM/Scl overlap syndrome is often reported as being a more benign and chronic disease with limited cutaneous involvement [19].

With respect to therapeutic modalities, the inflammatory features of PM/Scl patients respond to low to moderate doses of corticosteroids [20–22]. The frequency of anti-PM/Scl aab also appears to vary between different ethnic groups, as they were not found at all in a large series of SSc patients (n=275) from Japan [23] and HLA-DRB1*15/*16 and HLA-DQA1*0101 appear to have a protective effect against developing anti-PM/Scl aab [1,24].

With an increasing number of techniques and assay platforms designed to test for anti-PM/Scl reactivity, the availability of an international anti-PM/Scl reference serum provided by the Serology Committee of the IUIS/WHO/Centres of Disease Control (CDC) (http://www.autoab.org/) through the auspices of CDC is an important step towards standardization. However, this particular serum sample represents an atypical anti-PM/Scl sample as it is negative or weak positive by most assays using recombinant PM/Scl-100, but shows a strong nucleolar staining pattern in IIF on HEp-2 cells. In addition, this sample was negative for PM/Scl-75c and other exosome components such as hCsl4, hRrp4, hRrp40, hRrp41, hRrp42, hRrp46 or C1D (unpublished data) by ELISA. Therefore, a yet unknown exosome component might be the main target of aab in CDC ANA 11. This serum was weak positive by PM1-Alpha ELISA (with 2.5 RU, cut-off=1.5 RU) and by *recom*Line Scleroderma IgG (Mikrogen, Neuried, Germany).

2. The PM1-Alpha peptide as an antigen for autoantibody detection

Once an amino acid sequence has been identified as a prevalent aab target, a synthetic peptide thereof represents an ideal antigenic substrate for immunoassays because it can easily be produced in high quality and quantity and lot to lot quality variation is minimized since the production is not dependent on highly variable biological systems as sources of antigens [25]. In addition, during peptide synthesis non-standard amino acids corresponding to post-translational modifications of the native antigen, which may be crucial for antigen recognition, can easily be incorporated [25–28]. This is of significant value for autoimmune research, diagnostics and therapeutics. Depending on the amino acid sequence, as well as the length and biochemical properties of the peptide, different coupling approaches can be used to immobilize the peptide on a solid phase (e.g. microtiter plate or microbeads). Several studies have reported a higher sensitivity of peptide based immunoassays compared with assay systems that are based on respective native or recombinant antigens [25,27]. These observations have raised the question, how can a peptide be a more sensitive antigen than the cognate full-length protein? For one thing, the sensitivity of an immunoassay is defined by its ability to differentiate between controls (i.e. healthy donors or disease controls) and the disease group of interest. Depending on the length and the amino acid sequence of a full-length antigen, aab cross-reactions with this protein can occur, which in turn reduces the ability to distinguish patients from unaffected individuals. False positive results can be eliminated by increasing the cut-off value, which in turn may produce false negative results of low titre specimens that actually contain disease specific aab. Another important issue is the surface exposure of the epitope: Contingent on the folding of the respective antigen, the targeted stretch of the epitope might be hidden inside the protein structure and could thus be hardly accessible for antibody binding. This problem can be abolished by the use of peptides rather than the respective full-length proteins [29]. Moreover, the high concentration of epitopes bound in a small area (e.g. microtiter wells) of peptide based assays is likely another important feature [29]. While peptide antigens on average comprise 15–50 amino acids, the ordinary full-length polypeptide antigen amounts to approximately 300 residues. When both antigens are used in the same absolute concentration, the molarity of a specified peptide epitope is significantly higher than the respective epitope of the full-length antigen. Since the first discovery of aab in patients with systemic autoimmune rheumatic diseases, a substantial number of disease specific autoantigens have been identified. The use of synthetic peptides comprising autoreactive epitopes presents unique challenges and has to be individually evaluated for every aab system [29]. Although significant efforts are being made to standardize aab assays, significant variations can be observed among different methods and commercial kits [30], which can be attributed in large part to the differing antigens used in the immunoassays. The variation among different tests is most pronounced for assays with complex antigens. Therefore, synthetic peptides as autoantigenic substrates may facilitate the desired standardization of aab assays.

The identification of the major PM/Scl epitope, namely PM1-Alpha emerged in 1992 when the PM/Scl-100 protein was cloned by two independent groups [1,6,7,29]. Using recombinant protein fragments, the major immunoreactivity was localized to the N-terminal third of the antigen. Some years later, the major antigenic region was narrowed to amino acid 229-244 [1,29]. In 2000, the prime reactivity of anti-PM/Scl-100 seropositive sera was localized to amino acids 231-245 of PM/Scl-100 using the SPOT technology [1,29,31]. Based on this study, a local alpha-helical structure was proposed for the major PM/Scl-100 epitope. Although the fine-specificity of sera with anti-PM/Scl reactivity was different, 14/14 sera showed reactivity to the major epitope [31]. Therefore, this peptide called PM1-Alpha was used to develop an ELISA to detect anti-PM/Scl antibodies. A pilot study found reactivity to this peptide in 55% of PM/Scl, 13.2% of SSc, 7.5% of PM and 1.7% of unrelated controls, with good agreement with recombinant PM/Scl-ELISA data [27]. Of particular interest, the agreement between the ELISA with PM1-Alpha and PM/Scl-100 was better in the group of PM/Scl overlap syndrome patients compared to the entire patient cohort [27]. This suggests that antibodies to PM1-

Alpha show higher correlation to the overlap syndrome than aab to other epitopes of PM/Scl-100.

3. Anti-PM1-Alpha antibodies in disease and serologically preselected serum cohorts

Anti-PM1-Alpha reactivity was recently analysed in a group of anti-PM/Scl positive sera as defined by conventional methods for the detection of anti-PM/Scl antibodies including IIF, IB and ID. The results were compared to the findings derived from ELISA with recombinant PM/Scl-100 and PM/Scl-75c [33] (Fig. 1). The discrimination between sera with PM/Scl reactivity and controls was better using the PM1-Alpha ELISA (area under the curve, AUC = 1.0) compared to the PM/Scl-100 (AUC = 0.98) and PM/Scl-75c ELISA (AUC = 0.85) as revealed by receiver operating characteristics (ROC) analysis. In the control group, 1/91 (1.1%) of the samples showed anti-PM1-Alpha reactivity. This sample showed a diffuse staining pattern in IIF and anti-RNP antibodies in confirmation assays. Although no nucleolar IIF staining pattern was observed, the sample also reacted with PM/Scl-75c and 100 by ELISA. This apparent controversy might be explained by the strong diffuse staining pattern which obscured the nucleolar staining. When the cut-off of each assay was adjusted to achieve 100% specificity, sensitivities of 97.5% were obtained for PM1-Alpha, 60.0% for PM/Scl-100 and 62.5% for PM/Scl-75c. The combination of PM/Scl-100 and PM/Scl-75c increased the sensitivity to 77.5%. Clear quantitative correlations could be observed between the PM1-Alpha and the PM/Scl-75c ($r = 0.50$; $p < 0.0001$), and the PM/Scl-100 ($r = 0.67$; $p < 0.0001$) results as well as between PM/Scl-75c and PM/Scl-100 ($r = 0.82$; $p < 0.0001$) according to Spearman [33].

In a recent international multi-centre study, anti-PM1-Alpha reactivity was found in 7.1% (35/495) of SSc patients without significant differences among the three contributing centres [34]. No statistically relevant correlation was observed between anti-PM1-Alpha and clinical features such as muscle, skin, heart or lung involvement. The most prevalent clinical features of anti-PM1-Alpha seropositive SSc patients are RP (100%), telangieactasias (52.2%), oesophageal involvement (39.1%) and digital ulcers (39.1%) (unpublished data). In good agreement with the frequency of anti-PM/Scl-100 (14–26%) in a number of studies [1,35], anti-PM1-Alpha antibodies were found in 37/145 (25.5%) of samples with a nucleolar IIF pattern. In another study, sera primarily from SLE patients ($n = 16$) were reactive with both dsDNA (tested by Crithidia luciliae immunofluorescence test CLIFT) and PM/Scl (tested by ID) [14]. When using state of the art diagnostic assays including a commercial CLIFT nDNA assay and the PM1-Alpha ELISA, the number of double positive samples (2/7) significantly decreased [16].

When all studies are combined, 1661 sera from SSc patients were tested for anti-PM1-Alpha reactivity. The prevalence was 7.9% without significant differences among five different centres. Anti-PM1-Alpha reactivity was also observed in 5/414 (1.2%) SLE patient sera [1,16]. A meta-analysis of anti-PM1-Alpha aab is shown in Table 1. Anti-PM1-Alpha antibodies have been found in sera that also had antibodies to Ro52, Jo-1, Scl-70, CENP and Ku. However, no clear association with any one of these aab was found [27]. In a recent study, Kaplan-Meiers analysis of a cohort of 294 SSc patients showed that the survival rate in anti-PM1-Alpha positive patients had no significant difference when compared to the entire cohort but compared to anti-CENP positive patient group the survival was longer, but shorter for those with anti-topo I antibodies [34]. This might be inconsistent with the findings of Steen in 2005 [19] where it was reported that the survival of anti-PM/Scl positive patients appeared to be longer compared to anti-CENP aab positive patients during the first years of observation in a cohort of patients with limited SSc. However, after 10 years the survival of anti-CENP positive patients was higher than that of anti-PM/Scl positive patients. This putative controversy might be explained by the different detection method or by the different patient cohorts. In the study by Steen, anti-PM/Scl antibodies were detected by ID and were found in 36/1432 (2.5%) of all SSc patients, in 2% of diffuse SSc and in 7% of limited SSc. Thus the number of anti-PM/Scl positive patients with limited SSc studied in the Kaplan-Meier analysis was relatively small compared to the anti-CENP aab positive group. Further data on more than 900 SSc patients tested for anti-PM1-Alpha will be derived

Table 1
Meta-analysis of anti-PM1-Alpha autoantibodies in disease cohorts

	Mahler et al. [27]	Mahler et al. [34]	Mahler et al. [16]	Santiago et al. [40]	Mahler et al. unpublished	All
PM/Scl	22/40 (55.0)	na	na	na	na	22/40 (55.0)
SSc	27/205 (13.2)	23/495 (7.1)	na	17/242 (7.0)	64/719 (8.9)	131/1661 (7.9)
lSSc	na	na	14/204 (6.9)	na	na	14204 (6.9)
dSSc	na	na	4/41 (9.8)	na	na	4/41 (9.8)
PM	3/40	na	na	na	na	3/40 (7.5)
DM	na	na	na	na	na	Na
SLE	3/114 (2.6)	na	2/300 (0.7)	na	na	5/414 (1.2)
RA	0/69 (0.0)	na	na	na	na	0/69 (0.0)
MCTD	0/6 (0.0)	na	na	na	na	0/6 (0.0)
UCTD	0/10 (0.0)	na	na	na	na	0/10 (0.0)
HCV	2/48 (4.2)	na	na	na	na	2/48 (4.2)
Organ specific	0/23 (0.0)	na	na	na	na	0/23 (0.0)
HD	0/4 (0.0)	na	na	na	na	0/4 (0.0)

Abbreviations: DM = dermatomyositis; dSSc = diffuse systemic sclerosis; HCV = hepatitis C virus; lSSc = limited systemic sclerosis; MCTD = mixed connective tissue disease; n.a. = not analysed; HD = healthy donors; PM/Scl = polymyositis/scleroderma overlap sysndrom; PM = polymyositis; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; UCTD = undifferentiated connective tissue disease.

from studies performed by the Canadian Scleroderma Research Group.

Whether the high sensitivity of the PM1-Alpha ELISA accounts for the missing clinical associations (i.e. with muscle involvement) is rather unlikely as the correlation between PM1-Alpha ELISA and other methods for the detection is high. However, further research is mandatory to address this question. Of interest, anti-PM1-Alpha reactivity was also observed in apparently ANA negative samples [34]. This observation has been reported for other aab before such as anti-ribosomal P, anti-Ro or anti-Jo-1 aab [36–38]. In a recent study, less than 50% of anti-ribosomal P positive samples demonstrate no cytoplasmic staining pattern in IIF and thus no evidence for the presence of anti-ribosomal P antibodies [32].

Recently, when a line immunoassay (LIA) was used to test SSc patients (n=280) for aab to PM/Scl-75c (expressed in insect cells) and PM/Scl-100 (expressed in *E. coli*), 10.4% and 7.1% were positive for PM/Scl-75c and PM/Scl-100, respectively. Although associations of the PM/Scl-75c antigen with lung fibrosis and myositis overlaps were reported [39], further studies are needed to verify these results as well as comparing the new LIA with the PM1-Alpha ELISA.

4. Conclusions

After the identification of PM1-Alpha in 2000, several studies have shown the high diagnostic accuracy of immunoassays based on this peptide. In independent investigations it was shown that PM1-Alpha is superior to recombinant PM/Scl-100 and PM/Scl-75c for the identification of sera with reactivity to the PM/Scl complex. The data indicate that the PM1-Alpha peptide antigen may become the assay of choice for the detection of anti-PM/Scl antibodies. Further studies are needed to further verify the available and promising data.

Take-home messages

- A single PM/Scl peptide termed PM1-Alpha can be used as sensitive and specific antigen for the detection of anti-PM/Scl antibodies.
- The PM1-Alpha peptide antigen seems to be superior to recombinant PM/Scl-75c and PM/Scl-100 for the identification of sera with anti-PM/Scl antibodies.
- Approximately 25% of samples with a nucleolar staining pattern in indirect immunofluorescence show reactivity to the PM1-Alpha peptide.
- Anti-PM1-Alpha antibodies are present in the sera of 55% of PM/Scl overlap, in 7.9% of SSc, in 7.5% of PM but are rarely found in other diseases such as SLE.
- In multi-centre studies on SSc patients, anti-PM1-Alpha antibodies were not commonly associated with clinical features, such as myositis, interstitial lung disease or extent of cutaneous involvement.

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Evaluation of the efficacy and safety of pamapimod, a p38 MAP kinase inhibitor, in a double-blind, methotrexate-controlled study of patients with active rheumatoid arthritis

New therapies are under development to improve the efficacy of the treatment of rheumatoid arthritis. Monotheparies should promise to be good enough as traditional ones in improvement of disease course and symptoms. In a recent study, **Cohen SB et al, (Arthritis and rheumatism 2009; 60; 335-344)** intended to compare the efficacy and safety of pamapimod (a selective inhibitor of the alpha-isoform of p38 MAP kinase) as a monotherapy in comparison with methotrexate (MTX) treatment in adult patients with active rheumatoid arthritis (RA). Patients in a randomly assigned to 1 of 4 treatment groups and received 12 weeks of double-blind treatment. One group received MTX (7.5 mg/week with planned escalation to 20 mg/week), and 3 groups received pamapimod (50, 150, or 300 mg) once daily. The primary efficacy end point was the number of patients meeting the American College of Rheumatology 20% improvement criteria (achieving an ACR20 response) at 12 weeks. Secondary end points included ACR50 and ACR70 responses, change from baseline in the Disease Activity Score in 28 joints (DAS28), categorical analyses of DAS28/European League Against Rheumatism response, and change from baseline in each parameter of the ACR core set of measures. Safety monitoring included recording of adverse events (AEs), laboratory testing, immunology assessments, administration of electrocardiograms, and assessment of vital signs. As a result, patients assigned to receive MTX and pamapimod had similar demographics and baseline characteristics. At week 12, fewer patients taking pamapimod had an ACR20 response (23%, 18%, and 31% in the 50-, 150-, and 300-mg groups, respectively) compared with patients taking MTX (45%). Secondary efficacy end points showed a similar pattern. AEs were typically characterized as mild and included infections, skin disorders, and dizziness. Pamapimod was generally well tolerated, but the 300-mg dose appeared to be more toxic than either the 2 lower doses or MTX. The researchers concluded that pamapimod was not as effective as MTX in the treatment of active RA.

Regulatory T cells induced by GM-CSF suppress ongoing experimental myasthenia gravis

It was previously observed that treatment utilizing granulocyte-macrophage colony-stimulating factor (GM-CSF) had profound effects on the induction of experimental autoimmune myasthenia gravis (EAMG). In this study, **Sheng JR. et al (Clin Immunol 2008; 128: 172-80)** show that EAMG induced by repeated immunizations with acetylcholine receptor (AChR) protein in C57Bl6 mice is effectively suppressed by GM-CSF treatment administered at a stage of chronic, well-established disease. In addition, this amelioration of clinical disease is accompanied by down-modulation of both autoreactive T cell, and pathogenic autoantibody responses, a mobilization of DCs with a tolerogenic phenotype, and an expansion of regulatory T cells that potently suppress AChR-stimulated T cell proliferation in vitro. These observations suggest that the mobilization of antigen-specific Tregs in vivo using pharmacologic agents, like GM-CSF, can modulate ongoing anti-AChR immune responses capable of suppressing antibody-mediated autoimmunity.