

Preclinical studies with synthetic peptides in systemic lupus erythematosus

Gil Amarilyo¹, Bevra Hahn¹, Antonio La Cava¹

¹Department of Medicine, University of California Los Angeles, California, USA

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1. ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease that causes multi-organ damage and significant morbidity and mortality. Various efforts have been made to modulate the imbalanced immune responses in this disease. The manipulation of the immune system through the use of soluble synthetic peptides serving as antigenic epitopes, in repeated doses, has been shown to induce immune tolerance and to reduce the clinical manifestations of the disease in murine models. Although clinical trials in humans with the anti-DNA Ig peptide hCDR1 have failed, recent results from a clinical trial with another peptide, p140, have shown promise. This review provides an overview on the preclinical and translational work with synthetic peptides in SLE.

2. INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that is caused by the production of autoantibodies, deposition of immune complexes, and complement fixation resulting in tissue damage (1). SLE affects more than 300,000 people in the U.S.A. (2) and is much more common in women (ratio of 10:1 to males in the adult population). SLE may involve the skin, joints, kidneys, lungs, nervous system, serous membranes and other organs.

Despite major progress towards understanding the pathogenesis of the disease in recent years, the etiology of SLE is still unclear. Also, there is no cure for the disease. The main therapies use corticosteroids (which cause non-

specific immune suppression), cyclophosphamide (an agent that kills dividing cells), and mycophenolate mofetil (a calcineurin inhibitor that reduces proliferation of activated lymphocytes) (1). Mycophenolate mofetil is generally used for induction therapy in lupus nephritis and neuropsychiatric lupus (that are SLE complications that cause high morbidity and mortality), whereas steroids and mycophenolate mofetil are used more commonly, including as maintenance therapy for lupus nephritis (1).

Unfortunately, the treatment with the tumor necrosis factor (TNF)- α inhibitors - that have been successfully used in other autoimmune diseases such as rheumatoid arthritis and Crohn's disease - has been associated with high rates of infection in SLE patients, as well as increased quantities of anti-double stranded DNA (dsDNA) and anti-cardiolipin antibodies, (3, 4). At present, there is interest in suppressing interferon (IFN)- α , IFN- γ , interleukin (IL)-6 and IL-17 in patients with SLE, and such strategies are currently tested in clinical trials. Other biologic treatments in SLE, such as anti-CD20 antibodies (that deplete B cells) are used off-label, usually as second-line therapy. In regard to anti-CD20, despite a positive experience with in open trials (5), prospective randomized controlled trials in patients with active SLE (on glucocorticoids and additional immunosuppressants) showed no difference in responses in the SLE patients as compared to the placebo group (6).

3. SLE PATHOGENESIS

The pathogenesis of SLE relates to the dysregulation and the activation of both the innate and the adaptive immune systems (1). Physiologically, the innate immune system acts as the first line of defense against invading micro-organisms, while regulating the adaptive immune response (7). For example, antigen presenting cells (APC) such as dendritic cells (DCs) or macrophages of the innate immune system not only kill microorganisms through endocytosis or phagocytosis, but also process them into small antigenic peptides subsequently presented to the T cells of the adaptive immune system. Naïve T cells recognize peptide/major histocompatibility complex (MHC) complexes on the surface of APC through specific T cell receptors (TCRs) that, along with the binding of co-receptors and costimulatory molecules, transmit the first and the second signal respectively, which are required for the activation of effector T cells that initiate the immune response (8, 9). Throughout evolution, many mechanisms of immune tolerance have been developed to eliminate potential danger to the host. Schematically, immune tolerance is defined as an inability to respond to an antigen through the inhibition of lymphocyte activation (10). While central tolerance is maintained by the deletion of immature T cells that recognize self-antigens in the thymus, peripheral tolerance for T cells is achieved by several mechanisms including: (a) the induction of functional anergy (an inability to respond to the same antigen in a future encounter); (b) deletion of self-reactive T cells by activation-induced cell death (apoptosis); (c) ignorance to self-proteins that are presented too poorly to be recognized; (d) production of anti-inflammatory cytokines; (e) the

suppressive action of regulatory T cells (Tregs) (11). Failure of one or more of these peripheral tolerance mechanisms may lead to the onset of an autoimmune disease. In the case of SLE, there is T cell activation together with the activation of the humoral immune response that leads to the production of autoantibodies and a down-regulation of Tregs. Thus, a potential strategy to restore immune homeostasis in the disease would be to induce immune tolerance.

4. RATIONALE IN THE USE OF PEPTIDES IN SLE

In the early 60's it was shown that the administration of antigen in aggregated form leads to the generation of a productive immune response, whereas administration of the same antigen in a soluble monomeric form leads to tolerance or absence of immunity after re-challenge (12, 13). Thus, an antigen presented to T cells with an adjuvant leads to immunity, whereas the same soluble antigen administered without adjuvant leads to tolerance. Another condition to induce tolerance is to repeat the administration of antigens below the threshold required for a stimulation of the immune responses. Since T cells recognize antigens as peptide fragments, synthetic peptides can be used as desired antigens. These considerations have somehow laid rationale grounds in the use of peptides to induce tolerance in transplantation and in autoimmunity. The specific mechanisms that associate with the induction of tolerance after administration of soluble peptides are still little known, and may involve the induction of one or more mechanisms of peripheral tolerance (14). Moreover, peptides could promote the suppression of T cell responses to other epitopes within the same protein from which the peptide derives (intramolecular suppression) or to epitopes on separate proteins that are part of a larger antigen (intermolecular suppression). This phenomenon could be an important feature of antigen-induced suppression because, as shown in mouse models, it may override the "epitope spreading" whereby the initiating autoimmune response to an antigen expands with time to include additional responses to other antigens (15).

5. CONSIDERATIONS ON THE USE OF PEPTIDES IN SLE

As mentioned before, in SLE there is a loss of self tolerance, with activation of both innate and adaptive immune responses resulting in B cell production of various types of autoantibodies. Therefore, an approach to restore immune tolerance in this disease was to identify T cell epitopes serving as antigens that could elicit immune reactivity and to use their amino acid sequences for the development of synthetic peptide to administer for the induction of tolerogenic, rather than immunogenic, responses. In the case of SLE, however, problems have arisen in the search for suitable peptides. First, the complexity and the nature of the activation of the immune system in SLE against multiple antigens represents a challenge in quenching the cascade of events leading to the production of autoantibodies and pro-inflammatory cytokines. Moreover, there is large diversity among humans in terms of disease subtypes and the type of dominant

antigens in the disease. Therefore, a peptide that may promote tolerance in mice may not necessarily promote tolerance in humans, or it may do so in only a fraction of SLE patients. An additional problem is that when a peptide is used in a clinical trial, it is usually employed in an advanced stage of the disease, and it may thus affect autoimmune mechanisms less efficiently. Despite these limitations, the possibility to induce tolerance by using peptides in SLE has been tested in pre-clinical and clinical studies that are discussed in this review.

6. pCONSENSUS

pConsensus (pCons) is an artificial peptide that has been tested in (NZB \times NZW) F_1 (NZB/W) lupus-prone mice. NZB/W mice spontaneously develop elevated titers of anti-dsDNA antibodies that contain T cell determinants in their V_H regions (16). To design pCons, 439 12-mer and 15-mer peptides (representing the V_H regions of four different immunoglobulin (Ig)G2a or IgG2b anti dsDNA antibodies from nephritic NZB/W mice) were synthesized. Each peptide was tested for the stimulation of T cell proliferation *in vitro* and was defined as stimulatory when showing an ability to increase proliferation of syngeneic T cells of at least 3-fold (17). After 21% of the peptides were found to be stimulatory, a consensus algorithm was constructed to include the stimulatory amino acids in a 15-mer consensus peptide (pCons) with amino acids that bind to H-2^d, one of the MHC class II molecules expressed by NZB/W mice. As a negative control, another H-2^d-binding 15-mer peptide lacking most of the T-cell stimulatory amino acids (pNeg) was synthesized (17). pCons was tested as a tolerogen in NZB/W mice treated with 1 gram of pCons or pNeg once a month intravenously beginning at either 10 or 20 weeks of age, continued throughout their lifetimes. pCons-treated mice had delayed onset of nephritis and their survival was significantly prolonged (17). In addition, the appearance of multiple autoantibodies characteristic of SLE such as anti dsDNA, anti-nucleosome, anti-cardiolipin, were all inhibited as well as the pro-inflammatory cytokine IFN- γ (18). Similar results were observed in mice that had already developed anti dsDNA IgG and proteinuria (17). The mechanisms of immune tolerance included the induction of two types of Tregs with distinct gene signatures (18-21). The first group consisted of expanded peptide-reactive CD4⁺CD25⁺Foxp3⁺ Tregs that suppressed anti dsDNA production *in vitro* (18). The second group included inhibitory CD8⁺ T cells (later defined as CD8⁺ Tregs) expressing Foxp3 and secreting transforming growth factor (TGF)- β , that were found to suppress anti dsDNA and IgG production both *in vitro* and *in vivo* (19, 20). CD8⁺ Tregs from pCons-tolerized mice were resistant to apoptosis, perhaps related to upregulation of the anti-apoptotic molecule bcl-2, and expressed low levels of programmed death-1 (PD-1) (21). Also, the silencing of Foxp3 in CD8⁺ Tregs from NZB/W immunized mice reduced the expression of PD-1 (22). Of note, blocking the PD-1/PD-1 ligand 1 (PD-L1) pathway in otherwise untreated NZB/W mice promoted tolerance and inhibited SLE (23). However, in mice tolerized with pCons, administration of anti-PD-1 abrogated tolerance. Those mice failed to generate Foxp3-expressing CD8⁺ Tregs,

and those cells lost their ability to suppress effector T cell proliferation (23).

When pCons was tested in preliminary translational studies on human peripheral blood mononuclear cells (PBMC) from SLE patients, an expansion of Tregs was observed in seropositive SLE patients but not in healthy controls or in seronegative SLE patients (24).

7. hCDR1

The peptide hCDR1 is based on the sequence of the complementarity determining region (CDR)1 of a human monoclonal anti-DNA autoantibody that bears the 16/6 idiotype (16/6Id) found on anti-DNA antibodies of about two thirds of SLE patients (25). Administration of hCDR1 in an immunogenic regimen to wild-type healthy mice induced lupus-like disease (26). In both Id 16/6 induced lupus, and in the spontaneous lupus model of the NZB/W mice, treatment with ten weekly subcutaneous injections of hCDR1 (50 to 200 μ g per mouse), prolonged survival and improved leukopenia, proteinuria and deposition of renal immune complexes (27). Disease markers analysis revealed not only reduction of anti dsDNA titers but also a reduction in pro-inflammatory cytokines such as INF- γ , IL-1 β , TNF- α , and upregulation of the anti-inflammatory cytokine TGF- β (27). Relatively similar mechanisms of induction of immune suppression were found for pCons and hCDR1, namely an expansion of Tregs and CD8⁺ Tregs (28). The depletion of CD8⁺ Tregs caused clinical and laboratory deterioration in hCDR1-treated mice (28). CD4⁺ Tregs were increased up to 40% compared to control peptide-treated mice, and the CD4⁺ T cells expressed higher levels of TGF- β and Foxp3 (28, 29). In contrast to the CD8⁺ Tregs, CD4⁺ Tregs decreased the clinical manifestations and reduced autoantibody production upon transfer from hCDR1-treated mice into already diseased mice (28). The treatment of the mice with hCDR1 also reduced the rate of T cell apoptosis through the downregulation of the c-Jun NH2-terminal kinase (JNK) (30) as well as downregulation of the Fas-Fas ligand pathway (measured as diminished activation of caspases 3 and 8) (31, 32). In addition, hCDR1 inhibited TCR signaling by upregulating Foxj1 and Foxo3a, two negative modulators of T cells activation, leading to inhibition of NF- κ B activation and IFN- γ secretion (27). Finally, treatment with hCDR1 upregulated the anti-apoptotic molecule bcl-xL in Tregs, where it played a role in the induction of Foxp3 (33). The hCDR1 peptide affected also the humoral system by reducing the expression and secretion of B cell activating factor BAFF, also known as B Lymphocyte Stimulator (BLyS) (34). BAFF is elevated in lupus mice and in approximately half of patients. Of note, a BAFF inhibitor (belimumab) was superior to placebo in two prospective, randomized clinical trials in patients with SLE, which resulted in the approval of this biologic agent in the treatment of SLE (6, 35). Finally, the treatment with hCDR1 lowered the expression of MHC class II, CD80 and CD86 on DCs (36). Because of the above encouraging results, hCDR1 was tested in lupus-prone pigs, where the injection of hCDR1 showed clinical improvement and decreased

production of inflammatory cytokines and autoantibodies (37). In PBMC from lupus patients cultured with hCDR1, there was downregulation of IL-1 β , IFN- γ and IL-10 and upregulation of TGF- β gene expression, resulting in an increased number of functional Tregs (38). In a clinical trial on lupus patients, 5 patients were treated with hCDR1 and 4 patients were given placebo (39). A significant reduction in clinical disease activity measured by SLEDAI-2K and BILAG scores was observed in the hCDR1-treated group as compared to the control group. Treatment with hCDR1 also upregulated mRNA expression of both TGF- β and FoxP3 and downregulated the expression of IL-1 β , TNF- α , IFN- γ along with inhibition of BlyS and the pro-apoptotic molecules caspase-3 and caspase-8. The phase 1 clinical trial with hCDR1 (Edratide™) administered subcutaneously in weekly injection showed good safety profile and was well tolerated. However, a randomized, double-blind, placebo-controlled, parallel assignment phase 2 study with Edratide™ that enrolled 340 SLE patients from 12 countries did not meet its primary endpoint (40).

8. ANTI-NUCLEOSOME PEPTIDE

Antibodies to nucleosomes, the basic unit of DNA packaging in eukaryotes, play an important role in SLE (41, 42). The inducing antigens probably derive from nucleosome-containing apoptotic blebs that have escaped apoptotic clearance, since nucleosomes can be found in the plasma of lupus patients and murine models in correlation with active disease. In addition, nucleosomes are found in glomerular deposits and in the skin of SLE patients. A close correlation also exists between nephritis and the presence of anti-nucleosome antibodies (42). Five major histone-derived autoantigens were found involved in lupus nephritis: H₁₂₂₋₄₂, H2B₁₀₋₃₃, H3₈₅₋₁₀₂, H4₁₆₋₃₉, and H4₇₁₋₉₄ (42). When lupus-prone SNF1 mice with glomerulonephritis were injected with anti-nucleosomal peptides, it was found that H4₇₁₋₉₄ peptide was the most effective peptide in preventing death from SLE (43). A brief treatment of pre-nephritic SNF1 mice with nucleosomal peptide not only delayed the development of lupus nephritis but also prolonged survival in old SNF1 mice with established glomerulonephritis (44). Moreover, there was tolerance spreading in the treated mice, since a single peptide from nucleosome could be recognized by multiple autoimmune T cells with diverse TCRs, and conversely, a single autoimmune T cell could recognize multiple nucleosomal peptides. H4₁₆₋₃₉ peptide was highly tolerogenic, being recognized by both autoimmune T and B cells, and possibly causing direct B cells inactivation (44). As in the studies with higher doses of pCons and hCDR1, treatment with very low dose of H4₁₆₋₃₉ peptide induced antigen-specific CD4⁺ and CD8⁺ Tregs (45, 46). It was also shown that plasmacytoid DCs captured H4₇₁₋₉₄ peptide and expressed it in a tolerogenic fashion. The adoptive transfer of these DCs to diseased mice induced antigen-specific CD4⁺ and CD8⁺ Tregs, increased TGF- β and decreased IL-17 production by T cells, and reduced IL-6 production by DCs (46).

9. ANTI-RNP PEPTIDE P140

The anti-ribonucleoprotein (RNP) antibodies bind to proteins containing U1-RNA. The U1-RNP particle is involved in splicing heterogeneous nuclear RNA into

messenger RNA. Anti-RNP antibodies are found in variable numbers of patients with SLE (and are the hallmark of the mixed connective tissue disease) (41, 47). An epitope that is present in the residues 131–151 of the spliceosomal U1-70K small nuclear ribonucleoprotein (snRNP) has been identified (48). This antigen is recognized by IgG and CD4⁺ T cells in MRL/lpr and NZB/W mice (48, 49). A synthetic analogue with phosphorylation on Ser140, named P140 (P140), reduced proteinuria and dsDNA antibody levels and prolonged the survival of treated lupus-prone mice (50). Moreover, treatment of lupus patients' PMBC with P140 inhibited T cell proliferation, increased Tregs numbers, and induced the secretion of IL-10 (51). Intramolecular tolerance spreading was shown by repeated administration of P140 into pre-autoimmune MRL/lpr mice, in which it transiently prevented the activation of effector T cells against other regions of U1-70K and other spliceosomal proteins (52). These observations led to clinical trials. In a recent phase IIb clinical trial, P140 (LUPUZOR™) was found to be safe and well tolerated, and significantly improved the Physician's Global Assessment (PGA) and SLEDAI scores of lupus patients who received three monthly subcutaneous doses of 200 μ g of the peptide plus standard of care (53–55).

10. ANTI-Sm PEPTIDE Smd1₈₃₋₁₁₉

The Smith (Sm) antigen is a nuclear non-histone protein. It was the first nuclear protein autoantigen to be described in SLE (56). The antigen to which anti-Sm antibodies bind is part of the snRNP system and plays a role in the splicing of precursor messenger RNA (47). Specifically, Sm autoantigens are composed of seven core proteins, B, D1, D2, D3, E, F and G (in the majority of the snRNP complexes) and form a heptamer ring - approximately 20 nm in diameter - with the snRNA (which are composed of U1; U2; U4-6; and U5) passing through the center. The anti-Sm antibodies bind to multiple antigens. Although anti-Sm antibodies that are detected in clinical laboratory have low sensitivity and can be found only in 10% of patients, they are highly specific (80–100%) for SLE (41, 47). Smd polypeptides are the most specific for SLE (47). The presence of these antibodies (namely, to anti-Smd1₈₃₋₁₁₉) has a specificity of 93% for SLE but also high sensitivity (compared to other Sm antibodies) (56). 70% of SLE patients have antibodies to Smd1₈₃₋₁₁₉, compared with less than 7% of control patients with other diseases or healthy individuals (56). T cells that recognize Smd1₈₃₋₁₁₉ peptide and favor B-cell production of anti-dsDNA were identified in NZB/W mice and in sera from patients with SLE (57–59). Peptide administration to NZB/W mice delayed production of autoantibodies, postponed the onset of lupus nephritis, and prolonged survival (60). Tolerance to Smd1₈₃₋₁₁₉ could be adoptively transferred by CD90⁺ T cells, which reduced T cell help for autoreactive B cells *in vitro*, and increased the number of Tregs (60).

11. CONCLUDING REMARKS

In the absence of a specific treatment for SLE, treatment with synthetic peptides represents an intriguing and challenging option. The results observed in lupus mice

Table 1. Summary of peptide therapy in murine models of SLE

Peptide name and sequence	Origin	Dose	Route	Peptide effects	Refs
pCons (FIEWNKLRFRQGLEW)	Synthetic, murine anti-dsDNA Ab	1000mg Q month throughout lifetime	IV	Delayed onset of nephritis Prolonged survival ↓ Anti dsDNA, anti-nucleosome, anti-cardiolipin, IFN-g, IL- 4 ↑ CD4 ⁺ Tregs, CD8 ⁺ Tregs	19-20
hCDR1 (GYYWSWIRQPPGKGEE WIG)	Human anti-DNA mAb	25–50 microg Q week for 10 weeks	SQ	Amelioration of renal and neurological manifestations Prolonged survival ↓ Anti dsDNA, IL-1b, TNF-a, IFN-g, IL- 10 ↑ TGF-b ↑ CD4 ⁺ Tregs, CD8 ⁺ Tregs ↓ T cell apoptosis ↓ BAFF (BLyS)	25-33
Anti nucleosome peptides H4 ₁₆₋₃₉ (KRHRKVLRDNIQGITKP AIRRIAR) H4 ₇₁₋₉₄ (TYTEHAKRKTVTAMDV VYALKRQG)	Nucleosomes	300 microg Q 2 weeks for 8 weeks	IV	Delayed onset of nephritis Prolonged survival ↓ Anti-dsDNA, anti-nucleosome, IgG, IFN-g, IL- 10 ↑ TGF-b ↑ CD4 ⁺ Tregs, CD8 ⁺ Tregs ↓ Th17 Cells	43-45
P140 (RIHMOVSKRSGKPRGYA FIEY)	Synthetic phosphorylated analog of 70K U1-RNP ₁₃₁₋₁₅₁	100 microg Q 2 weeks for 6 weeks + another dose after 4 weeks	IV	Delayed onset of nephritis Prolonged survival ↓ Anti-dsDNA, ↑ CD4 ⁺ Tregs	47-49
SmD1 ₈₃₋₁₁₉ (VEPKVKSKKREAVAGR GRGRGRGRGRGRGR GRGPRR)	snRNP	0.6-1mg Q month throughout lifetime	IV	Amelioration of renal manifestations Prolonged survival ↓ Anti-dsDNA, anti-SmD1 ₈₃₋₁₁₉ , IFN-g, IL- 4, IL-10 ↑ TGF-b ↑ CD4 ⁺ Tregs	59

Table 2. Peptides in translational and clinical studies in SLE patients

Peptide name and sequence	Type of experiment	Dose	Route	Peptide effects	Refs
pCons (FIEWNKLRFRQGLEW)	Translational, PMBCs			↑ CD4 ⁺ Tregs	23
hCDR1 (GYYWSWIRQPPGKGEE WIG)	Translational, PMBCs			↓ Anti-dsDNA, IL-1b, TNF-a, IFN-g, IL- 10 ↑ TGF-b ↑ CD4 ⁺ Tregs ↓ T cell apoptosis	37
hCDR1 (GYYWSWIRQPPGKGEE WIG)	Clinical trial	0.5,1.0 or 2.5mg Q week for 26 weeks	SQ	↓ SLEDAI-2K, BILAG scores IL-1b, TNF-a, IFN-g, IL- 10, BAFF (BLyS) gene expression ↑ TGF-b, FoxP3 gene expression ↓ caspase-3, caspase-8	38
P140 (RIHMOVSKRSGKPRGYA FIEY)	Translational, PMBCs			↑ IL- 10 ↑ CD4 ⁺ Tregs	50
P140 (RIHMOVSKRSGKPRGYA FIEY)	Clinical trial	0.2 or 1.0mg Q 2 weeks for 6 weeks	SQ	↓ SLEDAI-2K, PGA scores ↓ Anti-dsDNA, IgG	52, 53

(Table 1) and on PBMC from SLE patients (Table 2) failed confirmation in the clinical trials with hCDR1 but are still promising in the case of the trials with P140 peptide. In general, the induction of tolerance in humans may be more complicated than in mice because of the genetic diversity, different mechanisms and subsets of disease including multiple genetic and environmental causes, and the relatively advanced stage of the disease by the time treatment begins. In addition, dosing of peptides in oral or subcutaneous regimens is critical to the induction of tolerance, and dose-finding in clinical trials can be difficult because of a relatively large number of patients. Notwithstanding these considerations, continuous progress towards the understanding of the mechanism of the disease pathogenesis may allow future pre-screening of better

candidates for peptide therapy, possibly as combination therapy with the current therapeutic approaches.

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Send correspondence to: Antonio La Cava, David Geffen School of Medicine, University of California Los Angeles, 1000 Veteran Avenue 32-59, Los Angeles, California 90095-1670, Tel: 310-267-4975, Fax: 310-206-8606, E-mail: alacava@mednet.ucla.edu

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