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The Histone Peptide H4_{71–94} Alone Is More Effective than a Cocktail of Peptide Epitopes in Controlling Lupus: Immunoregulatory Mechanisms

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Abstract

Tolerance therapy with nucleosomal histone peptides H4_{71–94}, H4_{16–39}, or H1'_{22–42} controls disease in lupus-prone SNF1 mice. It would be clinically important to determine whether a cocktail of the above epitopes would be superior. Herein, we found that compared with cocktail peptides, H4_{71–94} monotherapy more effectively delayed nephritis onset, prolonged lifespan, diminished immunoglobulin G autoantibody levels, reduced autoantigen-specific Th1 and Th17 responses and frequency of T_{FH} cells in spleen and the helper ability of autoimmune T cells to B cells, by inducing potent CD8 Treg cells. H4_{71–94} therapy was superior in “tolerance spreading,” suppressing responses to other autoepitopes, nucleosomes, and ribonucleoprotein. We also developed an in vitro assay for therapeutic peptides (potentially in humans), which showed that H4_{71–94}, without exogenous transforming growth factor (TGF)- β , was efficient in inducing stable CD4⁺CD25⁺Foxp3⁺ T cells by decreasing interleukin 6 and increasing TGF- β production by dendritic cells that induced ALK5-dependent Smad-3 phosphorylation (TGF- β signal) in target autoimmune CD4⁺ T cells.

Keywords

Systemic lupus erythematosus; T cells; DC; peptides; tolerance; autoimmunity

Introduction

The goal of ideal therapy is to restore immune regulation in lupus. Although curbing inflammation is an essential first step, targeted downregulation of autoimmune cells must follow to prevent recurrence and cumulative damage to vital organs.

In lupus patients and mice prone to develop lupus-like disease [1], autoimmune Th cells that drive production of pathogenic anti-DNA autoantibodies [2,3] recognize certain peptides in nucleosomal histones, which are also recognized by autoantibody-producing B cells; and spontaneous priming of the Th cells to these particular immunodominant epitopes occurs in preclinical stages. By pepscan of overlapping synthetic peptides and mass spectrometry of naturally processed peptides eluted from major histocompatibility complex class II (MHCII) of lupus antigen-presenting cell (APC), five major autoepitopes for lupus-nephritis-inducing Th cells and autoimmune B cells of murine and human lupus were localized in histone regions, H1'_{22–42}, H3_{85–102}, H3_{115–135}, H4_{16–39}, and H4_{71–94} [4–6], and overlapping

epitopes were detected by others [7,8]. The disease-relevant peptide epitopes accelerate lupus nephritis upon immunization with adjuvants, but they delay progression or even reverse disease upon administration (tolerization) in soluble form, at high doses intravenously or intranasally or at low doses subcutaneously [4,5,9–12].

Subcutaneously administered, low-dose tolerance therapy in lupus-prone SNF1 mice with nucleosomal histone peptide epitopes, H4_{71–94}, H4_{16–39}, or H1'_{22–42}, diminished autoantibody levels in serum, delayed nephritis development, markedly prolonged survival, and especially reduced inflammatory cell reaction and infiltration in the kidney [11,12], which is a major complication of lupus [11–13]. These peptide epitopes containing both MHC class II and class I binding motifs, induced CD8⁺, as well as CD4⁺CD25⁺ Treg cell subsets that included autoantigen-specific Treg cells [11]. Both subsets of Treg produce and require transforming growth factor (TGF)- β for immunosuppression and are potent in blocking spontaneous SLE, as well as suppressing accelerated and severe lupus disease in vivo upon adoptive transfer [11]. Among the peptides given singly, H4_{71–94} showed the most beneficial result in SNF1 mice. To bring the peptide therapy to the clinic, it is important to determine whether a cocktail of the peptide epitopes would be superior to therapy with a single peptide and define the immunoregulatory mechanisms.

Methods

Mice

NZB and SWR mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Lupus-prone SNF1 hybrids were bred, and females were used, as approved by the Northwestern University Institutional Ethics Review Board.

Peptides

All peptides were synthesized by F-moc chemistry and their purity checked by amino acid analysis by the manufacturer (Chiron Mimotopes, San Diego, CA, USA).

Tolerance Induction with Very Low Doses of Peptides

For long-term experiments, serologically autoimmune, but pre-nephritic, 12-week-old SNF1 females (eight mice per group) with lupus were injected SC with H4_{71–94} peptide alone (0.37 nM per mouse) or trio cocktail peptides (H1'_{22–42}, H4_{16–39}, and H4_{71–94}, at a dose of 0.37 nM of each peptide per mouse, or 0.37 nM total combined dose of the trio cocktail peptides per mouse) in phosphate-buffered saline (PBS) every 2 weeks. Control group received only PBS. Results of trio cocktail peptide therapy with 0.37-nM dose of each peptide per injection are shown, but similar results were obtained with trio cocktail peptides administered at 0.37 nM total combined dose per mouse per injection (data not shown).

The mice were monitored weekly for proteinuria using Albustix (VWR International, West Chester, PA, USA). Sera were collected every month for determination of immunoglobulin G (IgG) anti-nuclear autoantibodies. Parallel batches of identically treated mice of each group were followed and killed at different time points for evaluation of renal lesions. To test immunological consequences of the tolerance therapy early on, another batch of 12-week-old SNF1 mice (five per group) was treated as above; they received a total of three injections of peptides at 2-week intervals. Ten days after the third injection, these short-term batches of mice were killed for analysis of autoimmune T and B cells and regulatory T (Treg) cells. The peptide therapy was started at 2 months of age in another group of mice with similar results (data not shown).

Autoantibody Quantitation

IgG class autoantibodies to single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), histone, and nucleosome (histone–DNA complex) were measured by enzyme-linked immunosorbent assay (ELISA) [5,11]. Subclasses of IgG autoantibodies were detected by ELISA using alkaline phosphatase (AP)-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (SouthernBiotech, Bir-mingham, AL, USA). One month after treatment, SNF1 mice (at 4 months of age) were bled for autoantibody measurement.

Cell Isolation

Total mononuclear cells, CD4⁺, CD8⁺ T, and CD90⁺ T cells from spleens were purified by using appropriate MACS isolation kits using magnetic-beads-conjugated antibodies specific to each antigen. CD4⁺CD25⁺ T cells were purified by mouse regulatory T-cell isolation kit according to the manufacturer (Miltenyi Biotec, Auburn, CA, USA). For dendritic cell (DC) isolation, spleens were injected with Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml collagenase (Worthington Biomedical, Lakewood, NJ, USA) and 50 µg/ml DNase (Roche Applied Science, Indianapolis, IN, USA) for digestion at 37°C for 45 min and then followed by washes in EDTA-containing buffer to prevent clumping as described [12]. CD11c⁺ DCs were purified by using magnetic-beads-conjugated antibodies specific to CD11c according to the manufacturer (Miltenyi Biotec). Purity of isolated cells was >90%.

In Vitro Stimulation of Purified DCs

We isolated DCs from low-dose H471–94-tolerized, trio-cocktail-peptide tolerized, or PBS-treated mice and stimulated DC (1×10⁶ cells per well of 96-well plates) with nucleosomes (0.3–30 µg/ml) or phosphorothioate CpG oligonucleotides 1585 (0.1–10 µg/ml) from Oligo Etc in 10% fetal bovine serum (FBS) containing DMEM. Culture supernatants were collected after 90 h for TGF-β1 or 72 h for interleukin (IL)-6. Amounts of IL-6 were measured by BD OptEIA™ ELISA set (BD Bioscience, San Jose, CA, USA). For TGF-β1, samples were acidified by addition of HCl at 20 mM for 15 min and were neutralized by NaOH, and then amount of TGF-β1 was measured by TGF-β1 Emax ImmunoAssay System (Promega, Madison, WI, USA).

Immunohistochemistry

One half of each kidney from tolerized or control mice were fixed in 10% formalin and paraffin-embedded. Paraffin sections were used for immunohistochemical analysis of Th17 infiltration, as described [12].

Enzyme-linked Immunosorbent Spot (ELISPOT) Assay

ELISPOT assay plates (Cellular Technology Ltd.) were coated with capture antibodies against interferon (IFN)-γ (BD Pharmingen) in PBS at 4°C overnight. Splenic T cells (1×10⁶) from treated mice were cultured with irradiated (3,000 rad) splenic APC (B cells, macrophages, and DC) from 1-month-old SNF1 mice in the presence of peptides or PBS control. Cells were removed after 24 h of incubation for IFN-γ or after 48 h for IL-17, and the reactions were visualized by addition of the individual anti-cytokine antibody (Ab) biotin and subsequent AP-conjugated streptavidin. Cytokine-expressing cells were detected by ImmunoSpot scanning and analysis (Cellular Technology, Shaker Heights, OH, USA).

Suppression Assay

Ten days after the final (third) injection of H471–94, trio peptides or PBS, CD4⁺CD25⁺ and CD8⁺ Treg cells (2.5× 10⁵) were isolated, and the ability of Treg cells to directly inhibit IFN-γ and IL-17 responses of unmanipulated SNF1 lupus T cells (1×10⁶) to nucleosomes

presented by APC was compared in ELISPOT assays. The ratio of Treg to lupus Th cells was 1:4 [12].

Induction of Treg Cells by Peptide In Vitro

Splenocytes or CD90⁺ cell and DCs were cultured in Roswell Memorial Park Institute (RPMI) 1640 media containing 10% FBS and 20 U/ml IL-2 in the presence of peptides. After 1, 2, 3, 4, and 9 days, cells were stained for foxp3 and latency-associated peptide (LAP) in CD4⁺CD25⁺ T cells and CD8⁺ T cells.

For induction of Treg cells by DCs, CD90⁺ T cells and DCs from 3-month-old unmanipulated SNF1 mice were cultured in the presence of various amounts of H471-94, trio cocktail peptides, and nucleosomes for 7 days and then stained for Treg cells as described in “Flow Cytometry.”

Flow Cytometry

Biotin- or phycoerythrin (PE)-conjugated Ab to LAP (R&D system, Minneapolis, MN, USA) was used for cell surface TGF- β . For foxp3 staining, cultured cells in the presence of peptides were stained with allophycocyanin-labeled anti-CD4, fluorescein isothiocyanate (FITC)-labeled CD25 (eBioscience, San Diego, CA, USA), and PercpCy5.5-labeled CD8 (BD Bioscience). After fixation and permeabilization of cells using Cytotfix/Cytoperm kit (eBio-science), cells were then stained with PE-labeled foxp3 Abs (eBioscience). We analyzed the induction of Treg cells by peptide in vitro by comparing percent of CD25⁺foxp3⁺ cells in CD4⁻ or CD8-gated cell population by using FACSCalibur and Cell quest program (BD Bioscience).

Intracellular Staining for p-Smad3 (TGF- β Signal)

Splenocytes (2×10^6 cells) were fixed by using fixation/permeabilization buffer (eBioscience) after culturing in the presence of peptides and harvesting at various time points. After Fc blocking on the cells, cells were stained with anti-p-Smad3 Ab (20 $\times$ dilution) from Cell Signaling Technology (Danvers, MA, USA) and FITC anti-rabbit IgG Ab (BioFix Laboratories, Owing Mills, MD, USA) according to the manufacturer's instruction (Cell Signaling Technology). Stained cells were analyzed in LSR II and Diva 2 and FlowJo programs (TreeStar; FlowJo, Ashland, OR, USA).

Blocking Endogenous TGF- β Signaling by ALK5 Inhibitor

Splenocytes or CD90⁺ cell and DCs were cultured in RPMI 1640 media containing 10% FBS and 20 U/ml IL-2 in the presence of peptides. To block endogenous TGF- β , 5 μ M SB-431542 (ALK5 inhibitor; Sigma-Aldrich) was added to the culture 1 h before adding peptides. After 4 or 7 days, cells were stained for Foxp3 in CD4⁺CD25⁺-T-gated cells in case of the cultures of CD90⁺ T cells and DCs or in CD4⁺-gated cells for cultures of splenocytes [14,15].

CFSE-Based Cell Proliferation Assays

T cells from H471-94- or trio-peptide-tolerized or PBS-treated SNF1 mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) using the Vybrant Cell Tracer Kit from Molecular Probes, Inc. (Eugene, OR, USA) following the manufacturer's protocol. CFSE-labeled CD90⁺ T cells (1×10^6) were co-cultured with H471-94-tolerized DC or PBS-treated DCs (2.5×10^5) in the presence or absence of Ag in crisscross combinations for 72 h and then stained with PercpCy5.5-labeled anti-CD4, allophycocyanin-labeled CD25 (BD Bioscience), and PE-labeled foxp3 Abs (eBioscience) or allophycocyanin-labeled CD8 (BD Bioscience). We performed flow cytometry to compare proliferation by gating on CD4⁺CD25⁺foxp3⁺

cells or on CD8⁺TGF- β ⁺ cells among CFSE-labeled cells by using Cyan ADP (Dako) with Summit software and FACS express 3 software (De Novo Software, Los Angeles, CA, USA).

Helper Assay

T cells (1×10^6 /well) from H4₇₁₋₉₄ single peptide, trio cocktail peptide, or PBS-treated SNF1 mice were co-cultured with unmanipulated or tolerized SNF1 splenic B cells (1×10^6 per well) in 96-well plates for 7 days, as previously described [2,16]. Respective B cell preparation was cultured alone to measure baseline autoantibody production. Culture supernatants were collected, freeze-thawed, and assayed by ELISA for Abs against dsDNA, ssDNA, histone, and nucleosomes (histone/DNA complex).

Statistical Analysis

Log rank test and the Student two-tailed *t* test were used. Results are expressed as mean $\pm$ SEM, unless noted otherwise.

Results

Low-Dose Tolerance Vaccine Therapy with Single H4₇₁₋₉₄ Peptide Epitope Prolongs Life Span by Delaying the Onset of Lupus Nephritis and Diminishing Autoantibody Levels More Effectively than a Trio of Peptide Epitopes (Cocktail)

We tested whether low-dose tolerance with peptide cocktail has a stronger effect on suppression of disease in lupus-prone SNF1 mice. We tolerized 3-month-old SNF1 female mice by subcutaneous injection of the mixture of two or three histone peptide epitopes (H1'₂₂₋₄₂ and H4₁₆₋₃₉; H1'₂₂₋₄₂ and H4₇₁₋₉₄; H4₁₆₋₃₉ and H4₇₁₋₉₄; or H1'₂₂₋₄₂, H4₁₆₋₃₉, and H4₇₁₋₉₄). Among the mixture of three epitopes (H1'₂₂₋₄₂, H4₁₆₋₃₉, and H4₇₁₋₉₄), named “trio cocktail peptides” here, each peptide individually was previously found to be effective as compared with other epitopes in delaying disease and prolonging animal’s life span; and the dose response of these epitopes was also worked out previously [9,11]. Therefore, herein, we compared single peptide (H4₇₁₋₉₄) with the trio cocktail peptide in low-dose tolerance therapy.

Both single and trio cocktail peptides could delay the onset of severe nephritis and prolong the animals’ life span. However, single peptide therapy was more effective in delaying onset of severe nephritis and prolonging animal’s life span than trio cocktail peptide therapy (Fig. 1a, b, log rank test: single therapy $P=0.0153$, trio cocktail therapy $P=0.0483$). After 16 weeks of treatment (mice at 30 weeks of age), 20% of mice in both H4₇₁₋₉₄- and trio-cocktail-peptide-tolerized groups showed severe nephritis while 80% mice in control group have severe nephritis (Fig. 1a). After 22 weeks of treatment, 20% of mice in H4₇₁₋₉₄-tolerized group and 60% of mice in trio-cocktail-peptide-tolerized group showed severe nephritis while 100% mice in control group have severe nephritis (Fig. 1a). At this time point, 100% of mice in H4₇₁₋₉₄-tolerized and 80% of mice in trio-cocktail-peptide-tolerized groups were alive, whereas only 40% of mice in the control group were alive (Fig. 1b, log rank test: single peptide $P=0.00248$, trio peptides $P=0.0414$). Although the difference between H4₇₁₋₉₄ single-peptide and trio-cocktail-peptide therapies was not significant, H4₇₁₋₉₄ single-peptide therapy prolonged animal’s life span more significantly than trio-cocktail-peptide therapy during ages of 7–13 months (log rank test, $P=0.0429$). One month after low-dose peptide therapy, we analyzed total IgG level in serum of H4₇₁₋₉₄ single-peptide-treated mice at about 4 months of age. The levels of IgG class anti-ssDNA, anti-nucleosome, and anti-histone autoantibodies were markedly reduced, up to 49%, 81%, and 91% in serum of H4₇₁₋₉₄-treated mice and 78%, 79%, and 93% in serum of trio-cocktail-peptide-treated mice, respectively (Fig. 1c, $P<0.02$ – 0.001). Levels of IgG class anti-dsDNA

in serum were not elevated at this early point, but anti-ssDNA and anti-nucleosome autoantibodies are more pathogenic in this lupus model [17, 18]. The distribution of IgG subclasses were not changed by low-dose tolerance therapy (data not shown, but similar to our previous study [11]). Similar results on nephritis development and autoantibody levels were obtained when therapy was started at 2 months of age in another group of mice (data not shown).

We also test whether single H4₇₁₋₉₄ single-peptide and trio-cocktail-peptide therapies can suppress autoantibody responses to other autoantigens, ribonucleoprotein (RNP), and RNA. H4₇₁₋₉₄ single-peptide therapy suppressed autoantibody responses to RNP and RNA, whereas trio-cocktail-peptide therapy did not suppress autoantibody response to RNP except RNA (Fig. 1d, $P<0.05-0.001$), showing more effective “tolerance spreading.” Overall, H4₇₁₋₉₄ monotherapy was more effective in vivo in reducing IgG autoantibodies in the two major autoantigenic particles critical in lupus pathogenesis, namely, nucleosomes and RNP (summarized in Table I).

For assessment of renal pathologic features at the earliest stages, before persistent proteinuria sets in, another group of 3-month-old mice was treated for 6 weeks. Kidney sections from peptide-treated or control mice were examined and graded for typical lesions of lupus glomerulonephritis such as glomerular enlargement, hypercellularity, crescent formation, mesangial thickening, glomerulosclerosis, and interstitial infiltration with mononuclear cells [5,9,19,20]. Six weeks after low-dose peptide treatments, kidney sections from control mice had an overall score of 2.25 ± 1.7 for nephritis, whereas H4₇₁₋₉₄- and trio-cocktail-peptide-treated groups showed 1.0 ± 0.2 and 1.4 ± 0.2 as overall score, respectively (Fig. 1e, f, $P<0.02-0.01$).

Trio Cocktail Peptide Therapy Could Suppress IFN- γ and IL-17 Responses of Autoimmune T Cells Only when Challenged with Lower Concentrations of Nucleosomes and Cognate Peptides

To analyze the immunological aspects, we tested nucleosome-specific Th1 and Th17 responses after tolerizing another batch of mice with single or trio cocktail peptides. Compared with PBS control treatment, H4₇₁₋₉₄ single peptide therapy could suppress both IFN- γ and IL-17 responses when challenged with doses up to 30 $\mu\text{g/ml}$ nucleosomes in vitro, but trio-cocktail therapy suppressed IFN- γ response only up to 10 μg and IL-17 response up to 3 $\mu\text{g/ml}$ nucleosome stimulations, respectively (Fig. 2a, b, $P<0.05-0.001$).

The T cells of lupus-prone mice are spontaneously primed to nucleosomal histone peptide epitopes early in life and respond to them when challenged ex vivo [4]. Since we had previously found that low-dose treatment with individual peptide reduced IFN- γ response against other histone peptide epitopes cross-reactively [11], we tested whether single peptide was better than cocktail in suppressing IFN- γ as well as IL-17 responses cross-reactively to the peptide epitopes. H4₇₁₋₉₄ single-peptide therapy resulted in the marked inhibition of both IFN- γ and IL-17 responses to cognate epitope as well as to other epitopes upon in vitro challenge with peptide ranging from 0.1 to 30 $\mu\text{g/ml}$ (Fig. 2c, d, $P<0.02-0.001$). In contrast to H4₇₁₋₉₄ single-peptide therapy, cocktail peptide treatment actually was associated with strong IFN- γ and IL-17 responses to each cognate peptide epitope at higher concentrations (3 to 30 $\mu\text{g/ml}$), even higher than control treatment (Fig. 2e, f, $P<0.001$). Trio cocktail peptides could suppress IFN- γ and IL-17 responses to each peptide only when challenged at low concentration (0.1 $\mu\text{g/ml}$).

Both of the H4₇₁₋₉₄ Single- and Trio-Cocktail-Peptide Therapies Reduced the Frequency of T_{FH} Cells in Spleen and the Helper Ability of Autoimmune T Cells to IgG Autoantibody-Producing B Cells

We analyzed whether both of the H4₇₁₋₉₄ single- and trio-cocktail-peptide therapies can reduce the frequency of T_{FH} in spleens of single-peptide-, trio-peptide-, or PBS-treated SNF1 mice by staining ICOS⁺PD-1⁺CD4⁺ cells [21] or by analyzing the level of BCL-6 mRNA [22]. Without stimulation with nucleosomes, H4₇₁₋₉₄ single- and trio-cocktail-peptide therapies reduced the percent of T_{FH} in spleens by 34% and 44%, respectively, as compared with PBS treatment in control mice (Fig. 3a, b, $P < 0.05$ – 0.01). We also analyzed the fold increase of BCL-6 mRNA in splenocytes of peptide or PBS-treated mice upon stimulation with nucleosomes in vitro. H4₇₁₋₉₄ single- and trio-cocktail-peptide therapies suppressed the increase of BCL-6 mRNA upon the stimulation by 69% and 57%, respectively, as compared with PBS treatment in control mice (Fig. 3c, $P < 0.01$ – 0.001).

We also compared whether trio-cocktail therapy can suppress the helper ability of Th cells to IgG autoantibody-producing B cells more effectively than single-peptide therapy using helper assays in vitro. CD90⁺ T cells and B cells plus APCs or T-depleted splenocytes from mice tolerized with single or trio cocktail peptides were co-cultured in the presence of various amounts of nucleosomes for 7 days and assessed for autoantigen-specific IgG levels in the culture supernatants. With 10 µg/ml nucleosome stimulation, H4₇₁₋₉₄ single-peptide treatment as compared with PBS control treatment of animal's cells markedly reduced IgG class autoantibodies to dsDNA, ssDNA, nucleosomes, and histones by 82%, 77%, 83%, and 98%, respectively. Trio-cocktail-peptide therapy also reduced the levels of IgG autoantibodies against dsDNA, ssDNA, nucleosomes, and histone by 55%, 94%, 55%, and 67%, respectively (Fig. 4, $P < 0.05$ – 0.001). H4₇₁₋₉₄ single-peptide therapy suppressed T helper function in IgG autoantibody production more significantly than trio-cocktail-peptide therapy, except for autoantibody to ssDNA (Fig. 4b, $P < 0.01$ – 0.001 ; and summarized in Table I).

H4₇₁₋₉₄ Single-Peptide Therapy Generates CD8⁺ Treg Cells with Stronger Suppressive Activity on Autoreactive Th17 Cells, but Trio-Cocktail-Peptide Therapy Generates Stronger CD4⁺CD25⁺ Treg Suppressing Th1 Autoreactivity

We also determined the direct suppressing ability of Treg cells on the IFN- γ responses to nucleosomes by culturing Treg cells from treated mice with T cells and APCs from 5-month-old unmanipulated SNF1 mice in the presence of various amounts of nucleosome (0.3–10 µg/ml, Fig. 5). CD4⁺CD25⁺ Treg cells from animals undergoing trio-cocktail therapy showed higher suppressive activity on nucleosome-specific Th1 cells than CD4⁺CD25⁺ Treg cells from H4₇₁₋₉₄ single-peptide therapy, showing even 36-fold higher suppression at 10 µg/ml nucleosome stimulation (Fig. 5a). CD8⁺ cells from trio-cocktail-peptide therapy animals also showed 1.3-fold higher suppressive activities on the Th1 cells at the 1 and 10 µg/ml nucleosome stimulation (Fig. 5b).

Since we observed that single-peptide and trio-cocktail-peptide therapies suppressed nucleosome-specific Th17 responses (Fig. 2b), we also determined direct suppressing ability of Treg cells on the IL-17 responses to nucleosomes. We could not detect a significant level of suppressive activity by CD4⁺CD25⁺ T cells from single-peptide- or trio-cocktail-peptide-treated mice on nucleosome-specific IL-17 responses (Fig. 5c), except for suppression of Th17 response by CD4⁺CD25⁺ Treg from H4₇₁₋₉₄ single-peptide-treated mice at 3 µg/ml nucleosome stimulation. CD8⁺ T cells from trio-cocktail-peptide therapy showed significant level of direct suppressive activity on nucleosome-specific Th17 response at a low challenge dose of 0.3 µg/ml nucleosomes. However, CD8⁺ T cells from H4₇₁₋₉₄ single-peptide therapy showed a 2.5-fold higher level of suppressive activity at a challenge dose of 10 µg/

ml nucleosomes, as compared with CD8⁺ T cells from trio-cocktail-peptide therapy (Fig. 5d, $P<0.001$).

H4₇₁₋₉₄ Single-Peptide Therapy Was Better than Trio-Cocktail-Peptide Therapy in Decreasing IL-6 Production, along with Increasing TGF- β 1 Production by DCs

Because we previously observed that low-dose peptide tolerance therapy increased TGF- β but decreased IL-6 production by DC upon stimulation with nucleosome or CpG [12], we compared the production of these cytokines by DCs from mice treated with H4₇₁₋₉₄ single peptide or trio cocktail peptides. DCs from single-peptide- or trio-cocktail-peptide-treated mice produced similarly increased amount of TGF- β upon stimulation with nucleosomes, but DCs from H4₇₁₋₉₄-tolerized mice produced significantly higher amount of TGF- β upon stimulation with CpG (Fig. 6a, b, $P<0.05$ – 0.001).

Both single-peptide and trio-cocktail-peptide therapies reduced IL-6 production by DCs upon stimulation with nucleosomes, but H4₇₁₋₉₄ single-peptide therapy inhibited IL-6 production by DCs more significantly than trio-cocktail-peptide therapy upon stimulation with CpG (Fig. 6c, d, $P<0.02$ – 0.001).

In Vitro Test for Tolerogenicity of Peptide Epitopes

We tested whether single or trio cocktail peptides can expand CD4⁺CD25⁺ Treg cells from tolerized mice by DCs in vitro. CFSE-labeled, whole T cells from low-dose H4₇₁₋₉₄-, trio-cocktail-peptide-, or PBS-treated control mice were cultured for 72 h with DCs from unmanipulated SNF1 mice in the presence of H4₇₁₋₉₄, trio cocktail peptides, nucleosomes, or PBS and then stained for cell markers. As shown in Fig. 7a, b, CD4⁺CD25⁺Foxp3⁺ T cells from H4₇₁₋₉₄-treated mice proliferated more than the cells from trio cocktail peptide therapy. Single peptide was more efficient in expanding CD4⁺CD25⁺Foxp3⁺ T cells by DC in vitro than trio cocktail peptides ($P<0.02$ – 0.001).

We also tested whether single H4₇₁₋₉₄ or trio cocktail peptides can induce CD4⁺CD25⁺Foxp3⁺ T cells from T cells of unmanipulated SNF1 mice. IL-2 and TGF- β can convert naïve CD4⁺CD25⁺ T cells to CD25⁺Foxp3⁺ suppressor cells [23], and tolerogenic DCs (pDCs) play a role in the induction of Treg cells, and peptides can induce tolerogenic DCs that produce an increased amount of TGF- β and an decreased amount of IL-6 [12,24]. Therefore, we cultured splenocytes from unmanipulated 3-month-old SNF1 mice in the presence of various amounts of peptides and IL-2 (20 U/ml) but without exogenous TGF- β for 9 days and then analyzed the percent increase of CD25⁺Foxp3⁺ T cells in CD4-gated cell population. H4₇₁₋₉₄ single peptide increased CD25⁺Foxp3⁺ T cells in CD4⁺ T cells (sustained foxp3 expression) up to 1.2-fold on stimulation of 1 μ g/ml H4₇₁₋₉₄ peptide, as compared with control peptide H2B₅₉₋₇₃ or PBS (32.4%), and trio cocktail peptides increased CD25⁺Foxp3⁺ T cells 1.1-fold as compared with PBS (Fig. 7c, d, $P<0.02$ – 0.001). To compare the induction of CD4⁺CD25⁺Foxp3⁺ T cells by peptides to whole autoantigen (nucleosome) stimulations, we culture CD90⁺ T cells and DCs from 3-month-old unmanipulated SNF1 mice in the presence of various amounts of H4₇₁₋₉₄, trio cocktail peptides, and nucleosomes. DC with H4₇₁₋₉₄ induced levels of CD25⁺Foxp3⁺ cells in CD4-gated cell population up to fourfold higher as compared with DC with nucleosomes at 1 μ g/ml concentration. DC with trio cocktail peptides increased levels of CD25⁺Foxp3⁺ T cells up to threefold higher as compared with DC with nucleosomes (Fig. 7e, f, $P<0.01$).

Because FoxP3 expression can occur transiently on T-cell activation, it was reassuring that the induction of FoxP3 was stable. Moreover, to establish that the Treg cells induced in vitro were real, we compared the percent of TGF- β associated LAP⁺ cells in CD4⁺CD25⁺ T cell population. Splenocytes were cultured with H4₇₁₋₉₄, trio cocktail peptides, and PBS in the

presence of 20 U/ml IL-2. We found that 0.1 μ g/ml peptide is the optimal concentration and that culture for 3 days is the optimal time point for LAP⁺ cell staining. Single H4₇₁₋₉₄ peptide and trio cocktail peptides increased LAP⁺ cells by 1.65- and 1.38-fold, respectively, as compared with PBS (Fig. 8a, b, $P<0.02-0.01$).

We could not detect a significant level of Foxp3 expression or LAP on CD8⁺CD25⁺ T cells in this culture system (data not shown).

We tested whether peptide epitopes generate tolerogenic signal (presumably by DC) to lupus CD4⁺T cells by TGF- β , by analyzing intracellular phosphorylation of Smad3 (p-Smad3) which is involved in TGF- β signaling [25]. Splenocytes from 3-month-old unmanipulated SNF1 mice were cultured in the presence of single H4₇₁₋₉₄ peptide or trio cocktail peptides or PBS, and then we analyzed the phosphorylation of Smad3. We observed a significant increase of p-Smad3 on days 1, 2, and 4 (Fig. 8c-e, $P<0.01-0.001$). Culture with H4₇₁₋₉₄ peptide for 1 h showed the highest increase of p-Smad3 in CD4⁺ T cells. Therefore, we compared the phosphorylation of Smad3 by peptide at 1- and 96-h time points. Single H4₇₁₋₉₄ peptide (1 μ g/ml) increased p-Smad3 by 1.8- and 1.5-fold at 1- and 96-h time points, respectively, whereas trio cocktail peptides increased p-Smad3 by 1.4- and 1.3-fold, respectively, as compared with PBS (Fig. 8d, $P<0.02-0.01$). Although 0.1 μ g/ml peptide induced LAP optimally, 1 μ g/ml peptide induced higher percent of p-Smad3⁺ cells.

To further confirm that endogenous TGF- β signal was involved in generation of the induced Treg cells in vitro, we blocked TGF- β signal by the ALK5 inhibitor SB431542. H4₇₁₋₉₄ peptide (1 μ g/ml) induced 7.4% Foxp3⁺CD4⁺CD25⁺ Treg cells in 7-day cultures of CD90⁺ T cells with DCs and 11.9% Foxp3⁺CD4⁺Treg cells in 7-day cultures of splenocytes, but ALK5 inhibitor blocked the induction of Foxp3⁺ Treg cells (Fig. 8f, g).

Discussion

Both IFN- γ -producing Th1 cells and IL-17- and/or IL-21-producing Th17 and T_{FH} cells are critical in helping the production of pathogenic autoantibodies [4,6,22,26] and development of lupus nephritis [4,12,27-30]. Nucleosomes from apoptotic cells provide the major autoimmunogen for pathogenic Th and B cells of lupus [18,31]. In lupus-prone SNF1 mice, autoimmune Th1 and Th17 cells are spontaneously pre-primed to nucleosomal histone peptide epitopes, and their respective cytokine responses can be detected upon challenge with the autoepitopes ex vivo, without creating any polarizing conditions or resorting to PMA and ionomycin stimulation [4,5,12]. Tolerance therapy with low doses of the critical histone peptide epitope/s diminishes autoantibody levels in serum, delays nephritis development, markedly prolongs survival, and especially reduces inflammatory cell reaction and infiltration in the kidney [11,12], which is a life-threatening complication of lupus [11-13]. Subcutaneously administered low-dose peptide tolerance induces tolerogenic plasmacytoid DC (pDC), which upregulates TGF- β and plays a critical role in generating potent Treg cells [12]. These Treg cells suppress autoantigen presentation and responses of pathogenic lupus Th1 and Th17 cells to nucleosomes and suppress autoantibody production by inhibiting nuclear autoantigen-specific Th and B cells in vitro and in vivo [11,12].

Herein, we found that compared with cocktail of peptides, H4₇₁₋₉₄ monotherapy more effectively delayed nephritis onset, prolonged lifespan, diminished pathogenic IgG autoantibody levels, reduced autoantigen-specific Th1 and Th17 responses and frequency of T_{FH} cells in spleen and the helper ability of autoimmune T cells to B cells, by inducing stronger CD8⁺ Treg in vivo. Interestingly, we observed that peptide-induced CD4⁺CD25⁺ Treg cells could not suppress nucleosome-specific Th17 response (Fig. 5c). It seemed that CD4⁺CD25⁺ Treg cells might function as inducer of Th17 cells or that these Treg cells

themselves might differentiate into Th17 cells [32] because, for their targets, we used CD90⁺ T cells cultured with APC or splenocytes from SNF1 mice with overt lupus (4–5 month old), which contain DCs producing a relatively high amount of IL-6 and low amount of TGF- β (Fig. 6) [12]; and we did not add any exogenous TGF- β . Nevertheless, autoantigen-specific Th17 responses were suppressed better by CD8⁺ Treg cells in contrast to the CD4⁺CD25⁺ T cells in our test system (Fig. 5d), and the CD8⁺ Treg cells were induced more efficiently by H4_{71–94} monotherapy. The critical peptide epitope/s (9–10 mer) for the CD8 Treg cells are nested within the larger (24 mer) H4_{71–94} peptide bearing both *class II and nested class I MHC motifs*, which are most likely processed further by APC for cross-presentation [11,12]. Competition for autoantigen processing and presentation when a mixture of peptides are used could explain the superiority of monotherapy as compared with peptide cocktail [33].

In other approaches, higher doses of peptides related to anti-DNA antibody V regions, such as pConsensus, or a peptide-matching germ line sequence of human VH4-family CDR1 could suppress lupus in BWF1 mice and induce Treg cells [34,35]. By comparison, only 1 μ g of histone peptide epitope is therapeutic in our low-dose tolerance of a mouse with lupus, which would be equivalent to a 1–2-mg dose range in a lupus patient, and in contrast to high-dose tolerance, which deletes autoantigen-specific T cells transiently, the low-dose tolerance therapy restores immunoregulatory cells that suppress both autoimmune Th and B cells, as well as autoantigen-presenting APCs [9,11,12,34,36,37].

Thus, among nucleosomal histone epitopes, H4_{71–94} is highly potent in low-dose tolerance therapy, by cross-reactively suppressing autoimmunity to other pathogenic epitopes as well as to whole nucleosomes and RNP, the two major target autoantigenic particles in lupus pathogenesis. Moreover, H4_{71–94} also can suppress lupus via nasal tolerance [10]. Importantly, H4_{71–94} also binds strongly to common HLA-DR alleles, and T cells reactive to this epitope can be detected in almost all lupus patients, as well as in SNF1 and BWF1 mice [5,6,9,38,39]. The peptide appear to be effective even when the autoimmune disease is already established. As shown here, in an in vitro test for screening tolerogenic peptides, H4_{71–94}, without addition of any exogenous TGF- β , was efficient in inducing stable Treg cells by decreasing IL-6 and increasing TGF- β production by DCs, which in turn induced Smad-3 phosphorylation (TGF- β signal) in target autoimmune CD4⁺ T cells. Remarkably, autologous hematopoietic stem cell transplantation-induced long-term remission in refractory lupus patients is dependent on the same type of potent Tregs generated by nucleosomal peptide therapy in lupus-prone mice [40].

Conclusions

H4_{71–94} single-peptide therapy controlled spontaneous lupus disease more effectively than a trio cocktail of peptides in SNF1 mice and showed higher tolerogenicity than trio cocktail peptides. H4_{71–94} is effective at low doses, generates long-lasting antigen-specific regulatory T cells that suppress pathogenic autoantibody production and lupus nephritis, and induces cross-reactive “tolerance spreading.” This epitope is also recognized by autoimmune T and B cells of all lupus patients tested irrespective of their HLA type. Therefore, H4_{71–94} single-peptide therapy might be suitable for restoring and maintaining immunoregulation after remission has been induced by more toxic or global immunosuppressive agents.

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Abbreviations

Treg	regulatory T cell
LAP	latency associated peptide

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Fig. 1. Beneficial effect of low dose tolerance therapy using single or trio cocktail peptides. Incidence of severe lupus nephritis (**a**) and percent survival (**b**) of lupus-prone SNF1 mice injected with single (H4₇₁₋₉₄), trio (H1'₂₂₋₄₂, H4₁₆₋₃₉, and H4₇₁₋₉₄) peptides, or PBS (8 mice/group). **c** Both single and trio peptide therapies markedly reduced the levels of IgG autoantibodies in the serum of treated mice. **d** Single (H4₇₁₋₉₄) peptide therapy reduced the levels of IgG autoantibodies against RNP and RNA in the serum of treated mice, but not trio cocktail peptide therapy except anti-RNA IgG autoantibody. Sampling for (**c**) and (**d**), SNF1 mice were bled after one month of treatment (at 4 mo of age) and were assayed for levels of anti-anti-dsDNA, anti-ssDNA, anti-nucleosomes, and anti-histone IgG autoantibodies, or levels of anti-anti-RNA, anti-RNP, anti-RNA plus RNP, and anti-DNA/histone IgG autoantibodies. IgG (mean $\pm$ SEM, OD at 405 nm, 5 mice/group). **e** With identical treatment regimens to those in (**c**), renal histologic features of lupus nephritis were evaluated. Single or trio peptide tolerance therapy lowered histopathology score of nephritis. **f** Representative histologic features of kidneys with identical treatment regimens to those in (**c**). Marked perivascular, interstitial, and glomerular infiltration of Th17 cells (arrow in the left panel) were detected only in the kidney of control-treated mice (original magnification, X200). **a-b**

(log rank test): ⁺, $P=0.0483$; ^x, $P=0.0414$; *, $P=0.0153$; ** $P=0.00248$. **c–d**: ⁺, $P<0.05$; ^x, $P<0.02$; *, $P<0.01$; **, $P<0.001$

Fig. 2.

Single low-dose peptide therapy decreased both IFN- γ and IL-17 responses to relevant peptide epitopes and nucleosomes by lupus T cells in ELI-SPOT, but trio cocktail peptide therapy decreased IFN- γ and IL-17 responses to only lower concentration of those autoantigens. **a–b** T cells from PBS, H4_{71–94}, or trio peptide treated SNF1 mice were challenged with nucleosomes in various concentrations and then analyzed for Th1 and Th17 responses. **c–d** Splenic T cells from PBS or H4_{71–94} treated SNF1 mice were challenged with another nucleosomal epitope (H4_{16–39}) and H4_{71–94} peptide therapy decreased both Th1 and Th17 responses also to this epitope. **e–f** Splenic T cells from PBS or trio cocktail peptide treated SNF1 mice were also challenged with H4_{16–39} peptide, but trio cocktail peptide therapy decreased Th1 and Th17 responses to only low concentration of H4_{16–39} peptide. IFN- γ and IL-17 responses are expressed in mean $\pm$ SEM positive spots per 1×10^6 T cells from three experiments (five mice per group). Baseline IFN- γ and IL-17 spots in lupus T cells and APC cultures without Ag were less than 10 spots per 1×10^6 . x, $P < 0.02$; *, $P < 0.01$; **, $P < 0.001$.

Fig. 3.

Both single H4₇₁₋₉₄ peptide and trio cocktail peptide therapies can reduce T_{FH} cells. **a** ICOS⁺PD-1⁺CD4⁺ T_{FH} cells were reduced in spleens of single H4₇₁₋₉₄ and trio peptide treated mice. Splenocytes from single peptide, trio cocktail peptide- or PBS-treated mice were stained for ICOS and PD-1 in CD4 gated cells without any stimulation *in vitro*. **b** Representative dot plot of ICOS⁺PD-1⁺ T cells in CD4 gated population. **c** Both single H4₇₁₋₉₄ peptide and trio cocktail peptide therapies suppressed the increase of BCL-6 mRNA upon stimulation with nucleosomes *in vitro*. Splenocytes from H4₇₁₋₉₄⁻, trio cocktail peptide- or PBS-treated mice were stimulated with nucleosomes (20 µg/ml) *in vitro* for 18 h and then analyzed for fold increase of BCL-6 mRNA by real time PCR. †, *P* < 0.05; *, *P* < 0.01; **, *P* < 0.001

Fig. 4.

H4₇₁₋₉₄ single peptide and trio cocktail peptide therapies suppress anti-dsDNA (a), anti-ssDNA (b), anti-nucleosomes (c), and anti-histone (d) autoantibody production by T and B cells in the nucleosome stimulated helper assay. Baseline levels of IgG autoantibodies produced by B cells cultured by themselves were: anti-dsDNA, 0.01 ± 0.005 ; anti-ssDNA, 0.05 ± 0.006 ; anti-nucleosome, 0.02 ± 0.001 ; anti-histone, 0.03 ± 0.008 mg/dL. +, $P < 0.05$; x, $P < 0.02$; *, $P < 0.01$; **, $P < 0.001$

Fig. 5.

Therapy with H4₇₁₋₉₄ alone generates CD8⁺ Treg cells with stronger suppressive activity on autoreactive Th17 cells, but trio cocktail peptide therapy generates stronger CD4⁺CD25⁺ Treg suppressing Th1 autoreactivity. Suppressive activity of T cell subsets from treated mice were assessed on IFN- γ and IL-17 responses of unmanipulated SNF1 lupus T cells to nucleosomes presented by APC in the ELISPOT assay (ratio of Treg: lupus Th=1: 4). **a** CD4⁺CD25⁺ Treg cells from trio cocktail peptide therapy showed 2.5 fold higher suppressive activities on nucleosome-specific Th1 cells than CD4⁺CD25⁺ Treg cells from single H4₇₁₋₉₄ therapy. **b** CD8⁺ cells from trio cocktail peptide therapy also showed 1.3 fold higher suppressive activities on the Th1 cells at 1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ nucleosome stimulation. **c** CD4⁺CD25⁺ Treg cells from either single or trio cocktail peptide therapy could not suppress nucleosome-specific Th17 responses, except for suppression of Th17 response by CD4⁺CD25⁺ Treg from H4₇₁₋₉₄ singly treated mice at 3 $\mu\text{g/ml}$ nucleosome stimulation. **d** CD8⁺ T cells from trio cocktail peptide therapy showed significant suppressive activity on nucleosome-specific Th17 response at 0.3 $\mu\text{g/ml}$ nucleosomes stimulation, but CD8⁺ Treg cells from H4₇₁₋₉₄ single peptide therapy showed higher suppressive activity at 10 $\mu\text{g/ml}$ nucleosome stimulation than CD8⁺ Treg cells from trio cocktail peptide therapy. +, $P < 0.05$; x, $P < 0.02$; *, $P < 0.01$; **, $P < 0.001$

Fig. 6.

DCs from H4₇₁₋₉₄ single and trio cocktail peptide tolerized mice produced markedly increased amount of TGF-β on stimulation of nucleosomes (a) or CpG DNA (b). Amount of TGF-β in culture supernatants of DCs were measured by ELISA. DCs from H4₇₁₋₉₄ single peptide- and trio cocktail peptide-tolerized mice produced markedly decreased amount of IL-6 on stimulation of nucleosomes (c) or CpG DNA (d). Amount of IL-6 in culture supernatants of DCs were measured by ELISA. +, $P < 0.05$; x, $P < 0.02$; *, $P < 0.01$; **, $P < 0.001$

Fig. 7.

In vitro, DCs expand more H4₇₁₋₉₄ specific CD4⁺CD25⁺ Treg cells from whole T cells of H4₇₁₋₉₄-tolerized mice than those of trio cocktail peptide-tolerized mice. **a** Results from three experiments were compared by histogram (CFSE dilution) for proliferated CD4⁺CD25⁺Foxp3⁺ T cells. Numbers represent the mean percentage of three separate experiments ($n=5$). **b** Representative dot plot of CD25⁺Foxp3⁺ T cells in CD4⁺ T cells from experiment (a). **c** In vitro, H4₇₁₋₉₄ and trio cocktail peptide therapies induced CD4⁺CD25⁺Foxp3⁺ T cells in splenocytes of unmanipulated 3 month old SNF1 mice. Splenocytes were cultured in RPMI 1640 plus FBS and IL-2 (20U/ml) for 9 days in the presence of peptides, and then analyzed for CD25⁺Foxp3⁺ T cell in CD4 gated population as compared to that of culture with PBS (32.4%). **d** Representative dot plot of CD25⁺Foxp3⁺ T cells in CD4⁺ T cells from experiment (c). **e** Comparison of the induction of CD4⁺CD25⁺Foxp3⁺ T cells by peptide epitope to that by whole autoantigen (nucleosome) stimulations. CD90⁺ T cells and DCs from 3 month old unmanipulated SNF1 mice were cultured in the presence of various amounts of H4₇₁₋₉₄, trio cocktail peptides, and nucleosomes for 7 days. DC pulsed with H4₇₁₋₉₄ induced up to 4 folds higher levels of CD25⁺foxp3⁺ cells in CD4 gated cell population as compared to DC fed with nucleosomes at 1 μg/ml concentration. (F) Representative dot plot of CD25⁺foxp3⁺ T cells in CD4⁺ T cells. +, $P < 0.05$; x, $P < 0.02$; *, $P < 0.01$; **, $P < 0.001$

Fig. 8.

Endogenous TGF- β dependent induction of LAP, Smad3 phosphorylation and expression of FoxP3 in CD4⁺ T cells by H4₇₁₋₉₄ or trio cocktail peptides in vitro. **a** Both H4₇₁₋₉₄ single and trio cocktail peptides (1 μ g/ml) in vitro induced LAP⁺CD25⁺CD4⁺T cells in splenocytes of unmanipulated 3 month old SNF1 mice. Splenocytes from unmanipulated 3 month old SNF1 were cultured in the presence of 20U/ml IL-2 and peptides for 7 days. Results (on 3rd day) from three experiments were compared by histogram. **b** Representative dot plot of LAP⁺CD25⁺ cells in CD4 gated population. Phosphorylation of Smad3 (p-Smad3) in CD4⁺ T cells by peptide. Splenocytes from 3 month old unmanipulated SNF1 mice were cultured in the presence of single H4₇₁₋₉₄ peptide or trio cocktail peptides (1 μ g/ml). **c** Significant increase in the phosphorylation of Smad3 on day 1, day 2 and day 4. Culture with H4₇₁₋₉₄ peptide for 1 h showed highest increase of pSmad3 in CD4⁺ T cells. **d** Comparison of Smad3 phosphorylation by peptide at 1 h and 96 h time points. Both H4₇₁₋₉₄ and trio cocktail peptides increased pSmad3 in CD4⁺ T cells in vitro. **e** Representative dot plots of p-Smad3⁺CD4⁺ T cells in CD4 gated population. Quadrants are demarcated based on each group's own isotype control staining of samples. Endogenous TGF- β is required in the

induction of Foxp3⁺CD4⁺Treg cells *in vitro*. ALK-5 inhibitor (TGF- β signal inhibitor) blocked in vitro induction of Foxp3⁺CD25⁺CD4⁺Treg cells by H4₇₁₋₉₄ peptide in the culture of CD90⁺ T cells and DCs for 4 days (f) and in culture of splenocytes for 7 days (g). In (g), CD4⁺ T cell gated population contained 14.9% (cultures with PBS DMSO=dotted line), 26.5% (cultures with H4₇₁₋₉₄ DMSO=thin red line) and 13.6% (cultures with H4₇₁₋₉₄ and ALK-5=thick blue line) Foxp3⁺CD4⁺ Treg cells. ^x, $P<0.02$; *, $P<0.01$; **, $P<0.001$

Table I

Summary for suppression of IgG autoantibody responses

AutoAbs	Treatment	H4 ₇₁₋₉₄ -tolerized		Trio-tolerized	
		In vivo	In vitro	In vivo	In vitro
Anti-dsDNA		+	++++	+	+++
Anti-ssDNA		+++	+++	+++	++++
Anti-nuc		++++	++++	++++	+++
Anti-histone		++++	++++	++++	+++
Anti-RNA		++	ND	++	ND
Anti-RNA/RNP		+++	ND	-	ND
zAnti-RNP		+++	ND	-	ND

+: 10–20% suppression; ++: 21–40% suppression; +++: 41–60% suppression; ++++: 61–80% suppression; +++++: 81–100% suppression; -: no suppression (stimulation); N, D.: non-determined. *In vivo*: IgG autoantibody levels in serum of mice; *In vitro*: IgG autoantibody levels in culture supernatants from helper assays.