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Major pathogenic steps in *human* lupus can be effectively suppressed by nucleosomal histone peptide epitope-induced regulatory immunity

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Abstract Low-dose tolerance therapy with nucleosomal histone peptide epitopes blocks lupus disease in mouse models, but effect in humans is unknown. Herein, we found that $CD4^+CD25^{high}FoxP3^+$ or $CD4^+CD45RA^+FoxP3^{low}$ T-cells, and $CD8^+CD25^+FoxP3^+$ T-cells were all induced durably in PBMCs from inactive lupus patients and healthy subjects by the histone peptide/s themselves, but in active lupus, dexamethasone or hydroxychloroquine unmasked Treg-induction by the peptides. The peptide-induced Treg depended on TGFβ/ALK-5/pSmad 2/3 signaling, and they expressed TGF-β precursor LAP. Lupus patients' sera did not inhibit Treg induction. The peptide epitope-induced T cells markedly suppressed type I IFN related gene expression in lupus PBMC. Finally, the peptide epitopes suppressed pathogenic autoantibody production by PBMC from *active* lupus patients to baseline levels by additional mechanisms besides Treg induction, and as potently as anti-IL6 antibody. Thus, low-dose histone peptide epitopes block pathogenic autoimmune response in human lupus by multiple mechanisms to restore a stable immunoregulatory state.
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Abbreviations: SLE, systemic lupus erythematosus; iTreg, induced regulatory T cells; H, histone; HSCT, hematopoietic stem cell transplantation; LAP, latency associated peptide; DEX, dexamethasone; HCQ, hydroxychloroquine; RA, retinoic acid; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3; RAPA, Rapamycin; APG, Apigenin; TSA, Trichostatin A; ODN, oligonucleotide.

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1. Introduction

Remission maintenance therapy for SLE that specifically targets pathogenic autoimmune cells could prevent recurrence and halt smoldering inflammatory damage. In SLE, cognate interactions between autoimmune T helper (Th) cells and B cells against epitopes from apoptotic nuclear autoantigens lead to production of pathogenic IgG autoantibodies [1–7]. The IgG autoantibodies form inflammatory immune complexes (IC) containing apoptotic cell derived DNA/RNA, and along with CD40L signals from hyperactive lupus T cells, stimulate lupus APC to produce IL-6, IFNα, and other amplifiers of the pathogenic response [8–15].

Five critical autoepitopes in apoptotic cell derived nucleosomes that are recognized by autoimmune T and B cells of *patients* and various mouse strains with SLE are in histone (H) regions, $H1'_{22-42}$, $H3_{82-105}$, $H3_{115-135}$, $H4_{16-39}$ and $H4_{71-94}$ [2,16–19], and these epitopes are promiscuously bound by all major MHC molecules. The peptides delay lupus progression and even restore normal life span, reducing proteinuria in mice with *established* renal disease upon administration in soluble form (tolerization) at high doses intravenously [20]. The peptides are also therapeutically effective when administered intranasally, or in low doses subcutaneously [21–24]. In such lupus-prone mice, tolerance therapy with *nanoMolar* doses of histone peptide epitope/s, which contain both MHC class II and class I binding motifs, induces expansion of potent, autoantigen-specific $CD8^+$, and $CD4^+CD25^+$ regulatory T cell (iTreg) cells which suppress via TGFβ the responses of lupus T cells to nuclear autoantigens, and reduce autoantibody production by inhibiting the T cell help; leading to normal survival span. The stable, autoantigen-specific Treg generated in vivo by the peptide therapy can also block accelerated disease upon adoptive transfer into lupus mice [22]. The therapy especially reduces inflammatory cell reaction in the kidney [22,23]; a major complication of human lupus [25,26]. Only 1 μg (0.34 nM) of the histone peptide epitope/s is effective in low-dose tolerance therapy of mice with lupus, which would be equivalent to 0.2 to 2 mg range in lupus patients. Moreover, similar to the potent CD8 iTreg generated by histone peptide therapy above, or by other autoantigens in mouse models [27–34], we found that in humans, autologous hematopoietic stem cell transplantation (HSCT) for severe lupus also generates identical $FoxP3^+$, LAP^{high} $CD103^{high}$ $CD8^{+TGF\beta\text{-producing}}$ regulatory T cells (CD8 iTreg), which repairs immunoregulatory deficiency in lupus to maintain patients in *true immunological remission* [19].

Because effect of the nucleosomal peptide epitopes in humans is *unknown*, we studied herein if the histone peptide epitopes have any immunoregulatory effects on lupus patients' autoimmune cells in vitro.

2. Materials and methods

2.1. Subjects

We enrolled 30 lupus patients (10 active and 20 in remission, 22–63 years) who fulfilled the American College of Rheumatology revised criteria for SLE [35], and 15 healthy subjects (23–57 years). Disease activity was scored by the Systemic Lupus Activity Measure index (SLAM) [36]. Patients with SLAM score <7 were considered inactive (remission), and those with SLAM $\geq$ 7 were considered active. Clinical demographic profile is shown in Table 1. The study was approved by the Institutional Review Board of Northwestern University.

2.2. Cytokines and reagents

IL-2 was purchased from R&D Systems (Minneapolis, MN), TLR9 ligand CPG-containing oligonucleotide (ODN) 2216 was from InvivoGen (San Diego, CA), and anti-IL6 (BD Pharmingen, San Jose, CA), SB-431542 (GlaxoSmithKline, King of Prussia, PA), dexamethasone (DEX), hydroxychloroquine (HCQ), retinoic acid (RA), 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), Rapamycin (RAPA), Apigenin (APG), and Trichostatin A (TSA) were all from Sigma (St. Louis, MO).

Table 1 Patient demographic: clinical and treatment status of SLE patients in the study. [a]

Patient code	Age/sex/race	SLAM score	Current treatment
1	51/F/C	4	HCQ, SSZ
2	60/F/AA	8	None
3	27/F/AA	7	MMF
4	40/F/H	1	HCQ, Vit D
5	22/F/AA	9	MMF, HCQ, Pred
6	41/F/AA	5	HCQ
7	41/F/C	0	AZT, HCQ
8	24/F/W	15	MMF
9	63/F/C	5	None
10	50/F/C	5	HCQ
11	25/F/AA	2	None
12	32/F/C	1	AZT, HCQ
13	29/M/C	5	MMF, HCQ, Pred
14	50/F/C	2	HCQ
15	63/F/C	3	HCQ
16	34/F/C	2	HCQ
17	43/F/AA	1	HCQ, Pred
18	28/M/H	17	MMF, HCQ, Pred
19	31/F/W	16	AZT, HCQ, Pred
20	40/F/C	5	HCQ
21	43/F/AA	9	HCQ, Pred
22	24/F/H	11	MMF, Pred
23	41/F/C	7	AZT, HCQ, Pred
24	49/F/C	1	HCQ
25	32/F/C	2	HCQ
26	63/M/C	5	HCQ
27	44/M/C	16	HCQ, Pred
28	41/F/C	4	HCQ
29	26/F/A	6	MTX, HCQ, Pred
30	40/F/W	12	Pred, HCQ, MMF

[a] Abbreviations: SLE, systemic lupus erythematosus; SLAM, Systemic Lupus Activity Measure; AA, African American; H, Hispanic; C, Caucasian; F, female; M, male; HCQ, hydroxychloroquine (Plaquenil); Pred., prednisone or steroids; SSZ, sulfasalazine; MMF, mycophenolate mofetil (CellCept); Vit D, vitamin D; AZT, azathioprine; MTX, methotrexate.

2.3. Peptides

Peptides were synthesized by F-moc chemistry and their purity was checked by amino acid analysis by the manufacturer (New England Peptide, Gardner, MA).

2.4. Cell culture

PBMCs from subjects were isolated with Ficoll-Paque Plus gradient. To test induction of Treg cells, PBMCs were cultured with low doses of histone peptide autoepitopes (4 μM of each peptide): $H1_{22-42}$, $H3_{82-105}$, $H3_{115-135}$, $H4_{16-39}$, and $H4_{71-94}$, as well as a control peptide called "control peptide A" [30] for 7–11 days, along with 50 U/ml of hIL-2 in RPMI 1640 (w/ L-glutamine, Gibco, Foster City, CA) supplemented with 10% heat-inactivated FCS, or in some experiment with 10% patient's autologous serum, and 1 mM sodium pyruvate, 1 X nonessential amino acids, 1 mM HEPES, 2 mM L-glutamine, 5×10^{-5} β-mercaptoethanol, and 100 U/ml penicillin/streptomycin. Then percent of stable CD4 and CD8 $FoxP3^+$ Treg cells were analyzed by flow cytometry. The positive responders in each experiment were identified when the % of FoxP3 positive cells in their peptide-treated cultures was ≥2 SD as compared to that in corresponding PBS-treated culture. Expression of the precursor of active TGFβ, latency associated peptide (LAP), and CD103 and CD39 in cultured T cells were also measured. In some experiments, to explore the effects of anti-inflammatory drugs or antibodies on FoxP3 expression, 1×10^{-5} M of dexamethasone (DEX), 25 μM of hydroxychloroquine (HCQ), 10 nM or 100 nM of retinoic acid (RA), 5 ng/ml of TGF-β, 5 μg/ml of anti-IL6 neutralizing antibody, 100 ng/ml of Rapamycin (RAPA), 20 ng/ml of 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), 12.5 μM of Apigenin (APG), or 0.25 μM or 0.1 μM of Trichostatin A (TSA) was also added to PBMCs cultured with the histone peptides or PBS control. To study the importance of TGFβ/ALK-5/Smad2/3 signaling pathway in Treg cells, 5 μM of ALK5 inhibitor (SD-431542) was added to PBMC cultures with peptide or PBS control.

To analyze the suppressive effect of single peptides or peptide cocktail on pathogenic autoantibody production by active lupus patients, their PBMCs (5×10^5 cells) in 200 μl/microwell were cultured in the presence of different low-dose peptide epitopes or peptide cocktails, or PBS in complete RPMI with 10% FCS and 50 U/ml IL-2 for 13 days. To exclude autoantibody production by B cells that were already preactivated in vivo, 120 μl of medium from each culture was replaced with fresh medium and corresponding peptides on day 5 without disturbing the cells at the bottom of the microwells. On day 13, production of IgG autoantibodies to double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and nucleosomes (histone/DNA complex) was quantitated in the culture supernatants. In subsequent text and figure legends, PBS means adding PBS instead of peptide in cell culture. The identity of peptide epitopes is abbreviated showing the first residue only: H1(22), H3(82), H3(115), H4(16) and H4(71) means low doses of indicated peptide added to the 13-day cultures. Peptide cocktail-1 (C1) means a mixture of H1(22), H3(115), and H4(16) at 1.5 μM of each; peptide cocktail-2 (C2) means a mixture of H1(22), H3(115), and H4(16) at 4 μM of each; peptide cocktail-3 (C3) means a mixture of H1(22), H3(82), and H4(71) at 1.5 μM of each; and peptide cocktail-4 (C4) means a mixture of the five peptides at1.0 μM of each. C1, C2, C3 or C4 was added in 13-day cultures as with the single epitopes. In some experiments, the low doses of anti-IL6 antibody (600 ng/ml) were used to detect it's suppressive function.

To measure the suppressive effect of peptide cocktail on type I IFN gene expression, PBMCs from inactive SLE patients who were taking very low doses of steroids were cultured for five days in the presence or absence of peptide cocktail-2 and then stimulated with the TLR9 ligand, CPG-containing oligonucleotide (ODN) 2216, for 4 h before the RNA were taken for qPCR. In some experiments, T and B cells were depleted with positive selection beads from PBMC before cell culture (STEMCELL Technologies Inc., Canada).

2.5. ELISA

IgG class autoantibodies to dsDNA, ssDNA and nucleosomes were measured as described [8]. Standard curves were obtained by serial dilutions of one active lupus patient's plasma with high IgG autoantibody titer. The addition of a mixture (cocktail) of the histone peptide epitopes to the lupus plasma did not affect the standard curves. Autoantibody levels are expressed as the absorbance value at 405 nm. The absorbance values of 1:1000 dilution of the reference patient's plasma were considered to be equivalent to 1 U/ml of anti-dsDNA, -ssDNA or anti-nucleosome autoantibodies for calculating the % inhibition by each peptide or cocktail [8].

2.6. Flow cytometry

Antibodies to stain following markers were used in different combinations: CD4-PerCP, CD4-PE-Cy7, CD8-APC, CD8-PerCP-Cy5.5, CD25-FITC, CD25-APC, CD45RA-V450, LAP-PE, CD103-PE, CD39-FITC, CD1c-FITC, CD80-Alexa Fluor700, HLA-DR-APC (BD Biosciences), FoxP3-PE and isotype control (eBioscience, San Diego, CA), Rabbit IgG or pSmad 2,3/goat anti rabbit-FITC (Cell Signaling, Danvers, MA). For intracellular staining, cells were treated with Fix/Perm Buffer set (eBioscience) and stained with anti-FoxP3-PE or phospho-Smad 2(Ser465/467)/Smad3 (Ser423/435) and then goat anti-rabbit-FITC, and corresponding isotype controls. Data collected on a LSR II flow cytometer (BD Biosciences) were analyzed with FlowJo (Tree Star, Ashland, OR).

2.7. Real-time quantitative PCR

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA) with DNase treatment, and 1.25 μg of total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Quantitative PCR was performed using TaqMan Gene Expression Master Mix and a 7300 Fast Real-time PCR System (Applied Biosystems). Expression of the tested genes was normalized relative to the levels of GAPDH. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ threshold cycle method.

2.8. Statistical analysis

Data analyses were performed using Prism 4.0 software (GraphPad software). Comparisons were performed by Student *t* tests. Results are expressed as mean ± SEM; *p* values < 0.05 were considered significant.

3. Results

3.1. Low doses of histone peptide epitopes by themselves durably induce FoxP3$^+$ Treg in vitro

In lupus-prone mice, the histone peptide epitope/s induce autoantigen-specific CD4$^+$CD25$^+$ and CD8$^+$ Treg cells in vivo, blocking lupus disease [22,23]. To detect whether the histone peptides can induce such Treg cells in humans, fresh PBMCs from active and inactive lupus patients and healthy subjects were cultured with low doses of the peptides (4 μM of each peptide): H1$_{22-42}$, H3$_{82-105}$, H3$_{115-135}$, H4$_{16-32}$, and H4$_{71-94}$, control peptide A or PBS for 3, 5, 7, 9 or 11 days, all with 50 U/ml IL2; the cells were cultured at 2.5 × 10^6/ml with 10% of FBS complete RPMI and then analyzed by flow cytometry. CD4$^+$CD25^{high}FoxP3$^+$ and the CD8$^+$FoxP3$^+$ cells began to increase after culture for 3 days, and up to 11 days tested. At day 7, the percentage of CD4$^+$CD25^{high}FoxP3$^+$ cells (Fig. 1) and CD8$^+$FoxP3$^+$ cells (Fig. 2) was significantly increased in PBMCs when cells were cultured with low-dose histone peptide, compared with control peptide A or PBS ($p < 0.01$). As shown in the left panels of Figs. 1A and 2A, for induction of FoxP3$^+$ Tregs, one peptide epitope may induce positive response in an individual patient but may be negative in another patient. Therefore, we summarized the Treg responder frequency as % of positive responders in right panels of Figs. 1A and 2A. A patient was considered to be a positive responder to a peptide if % of FoxP3 Tregs increased above 2 SD over its PBMC cultured without peptide (PBS). The peptide H1$_{22-42}$ induced the highest frequency (up to 80%) of FoxP3$^+$ Treg response in PMBCs from inactive lupus patients, followed by H3$_{82-105}$ and H4$_{16-39}$.

In humans, unlike mice, FoxP3 can be induced in naïve CD4$^+$FoxP3$^-$ T cells by activation, without suppressive activity. Only CD4$^+$CD25^{hi}FoxP3$^+$ T cells show Treg function in humans. Moreover, FoxP3$^+$ T cells are heterogeneous in humans; in fresh PBMCs, CD45RA$^+$FoxP3^{low} CD4 T cells are naïve Tregs (nTregs) and CD45RA$^-$FoxP3^{hi} CD4 T cells are considered effector Tregs (eTregs); and similar to CD25^{hi}CD4$^+$ cells, both of them are potently suppressive in vitro. Effector Tregs are reported to be poorly proliferative, when stimulated with antigen, whereas human nTregs can easily expand upon stimulation, and acquire eTreg phenotype as they upregulate FoxP3, CD25, and CD45RO (CD45RA$^-$) when stimulated with antigen [37]. We found that peptide epitopes significantly increased CD4$^+$CD45RA$^+$FoxP3^{low}, but did not increase CD4$^+$CD45RA$^-$FoxP3^{hi} cells (Fig. 1C), indicating that most of the Treg were newly induced by the peptide epitopes.

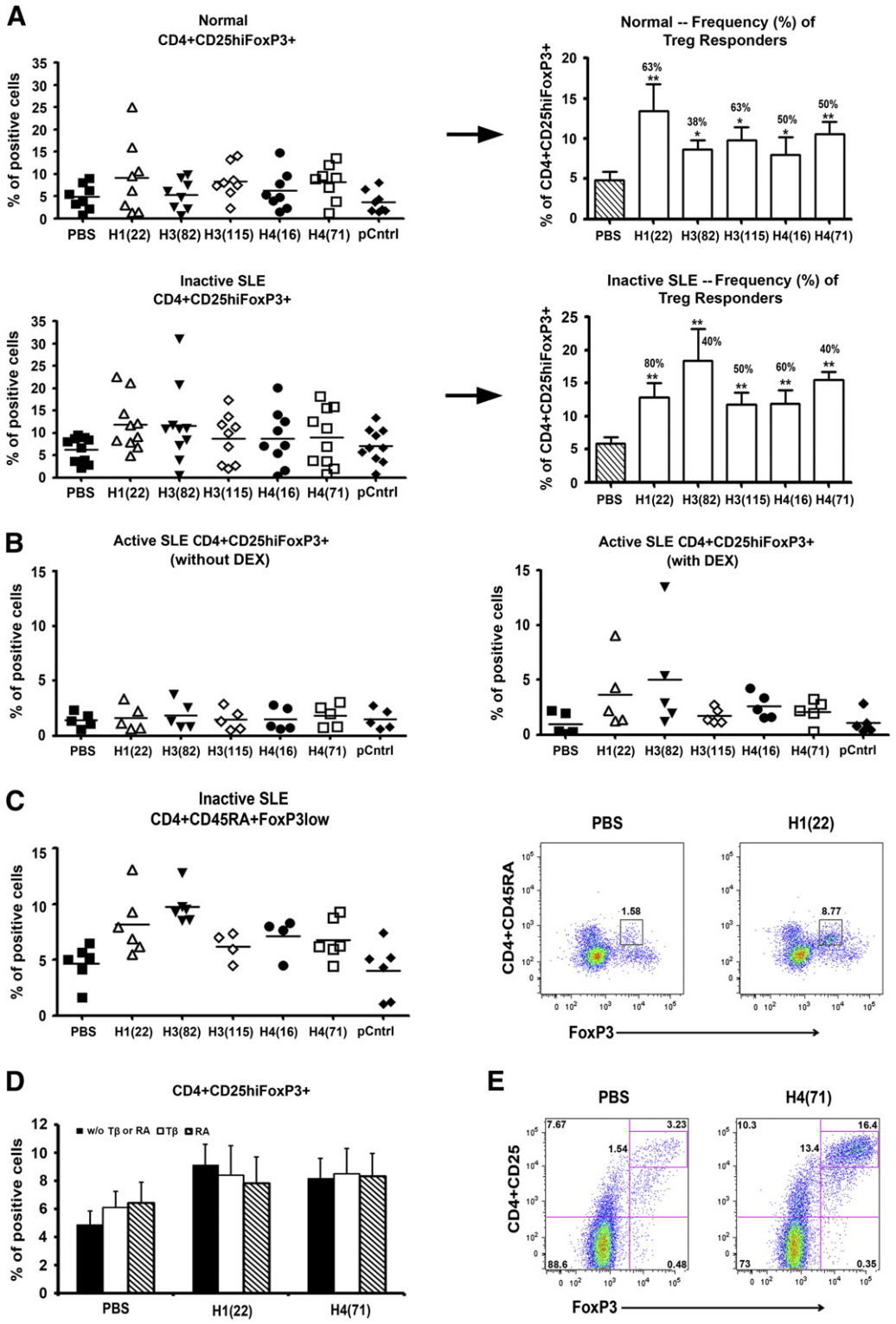
3.2. Effect of exogenous agents on Treg induction by the peptide epitopes

We tested whether patient's serum, which contains type I IFN-inducing inflammatory immune complexes [12,13], would block the FoxP3$^+$ Treg inducing effect. We supplemented the culture medium with 10% autologous serum of each patient instead of 10% FBS and compared with FBS supplemented medium, and found no significant differences, indicating that patient's serum could not block peptide-induction of FoxP3$^+$ Tregs (data not shown).

We tested whether conventional lupus drugs, such as dexamethasone (DEX) or hydroxychloroquine (HCQ) could enhance the FoxP3$^+$ Treg inducing effect of peptide epitopes. PBMCs from lupus patients were cultured with low-dose peptide in the presence or absence of DEX (1 × 10^{-5} M) or HCQ (25 μM) for 7 days. We had found that unlike inactive patients, in PBMC from *active* lupus patients, low-dose histone peptides have very weak FoxP3$^+$ Treg inducing effect (Figs. 1B and 2B, left panels), however, when cultured with DEX, FoxP3$^+$ Tregs increased significantly in the latter (Figs. 1B and 2B, right panels). HCQ also had enhancing effect on peptides inducing Tregs as DEX did. Compared to control cultures with HCQ and control peptide or PBS, HCQ plus the different peptide epitopes increased CD4$^+$CD25^{hi}FoxP3$^+$ Tregs by 2.2 to 5.5 fold and CD8$^+$FoxP3$^+$ Tregs by 1.9 to 4.9 fold in active SLE

Figure 1 Durable induction of CD4$^+$CD25^{hi}FoxP3$^+$ and CD4$^+$CD45RA$^+$FoxP3^{low} Treg cells in vitro by low-dose histone peptides. (A) Fresh PBMCs from healthy subjects and inactive SLE patients were cultured with histone peptide epitopes, control peptide or PBS, all in the presence of 50 U/ml IL2 for 7 days, and then stained for CD4, CD25, and FoxP3. Peptide epitope names are abbreviated in X-axis (see Material and methods). Y-axes show % of FoxP3 positive cells among viable T cells gated for being CD4$^+$CD25^{hi}; and each symbol represents data from one individual. pCntrl = control peptide A; horizontal lines indicate the mean. Right panels (next to →): The Y-axis here is the same as in the left panels, designating the % of CD4$^+$CD25^{hi} cells that were FoxP3 positive. Except for PBS, the other bars represent mean ± SD of the individual data points shown in the left panels, but for only the positive Treg responders to each peptide. These positive responders in each experiment were identified when the % of FoxP3 positive cells in their peptide-treated cultures was ≥2 SD as compared to that in corresponding PBS-treated culture. The numbers above the bars represent the frequency (%) of positive Treg responders for each peptide. *p* values of FoxP3$^+$ cells over control are indicated, $^{**}p < 0.01$, $^{*}p < 0.05$. n = 5 to 10. (B) Fresh PBMCs from active SLE patients were cultured with histone peptide epitopes and IL2, in the presence or absence of dexamethasone (DEX) for 7 days, and then stained for CD4, CD25, and FoxP3. (C) Fresh PBMCs of inactive SLE patients were cultured with histone peptides for 7 days and stained for CD4, CD45RA, and FoxP3. The right 2 panels showed the representative Dot Plots of CD4$^+$CD45RA$^+$FoxP3$^+$ cells induced by peptide H1$_{22-42}$. Panel (D) Shows the effects of TGF-β (Tβ) or retinoic acid (RA) on induction of CD4$^+$CD25^{hi}FoxP3$^+$ Treg cells by two of the peptide epitopes, n = 3. (E) Representative Dot Plots show Treg induction in PBMC after culturing with the peptide epitope H1$_{22-42}$ or H4$_{71-94}$ for 7 days: the two panels show gated CD4$^+$ T cells in PBMC for CD25$^+$FoxP3$^+$ cells.

PBMCs. Certain agents are known to enhance the effect of anti-CD3 and −CD28 induced Tregs in healthy subjects, such as the major vitamin A metabolite all-trans retinoic acid (RA), TGFβ, anti-IL6 antibody, the potent immunosuppressive drug Rapamycin (RAPA), the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), which can inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. Therefore, we also explored the effects of these drugs or antibodies on peptide inducing $FoxP3^+$ Tregs in lupus patients. Our results indicated that RA, TGFβ, $1,25(OH)_2D_3$, APG, and TSA did not show any enhancing effect in the induction of $FoxP3^+$ Tregs above and beyond that induced by low-dose histone peptide/s themselves



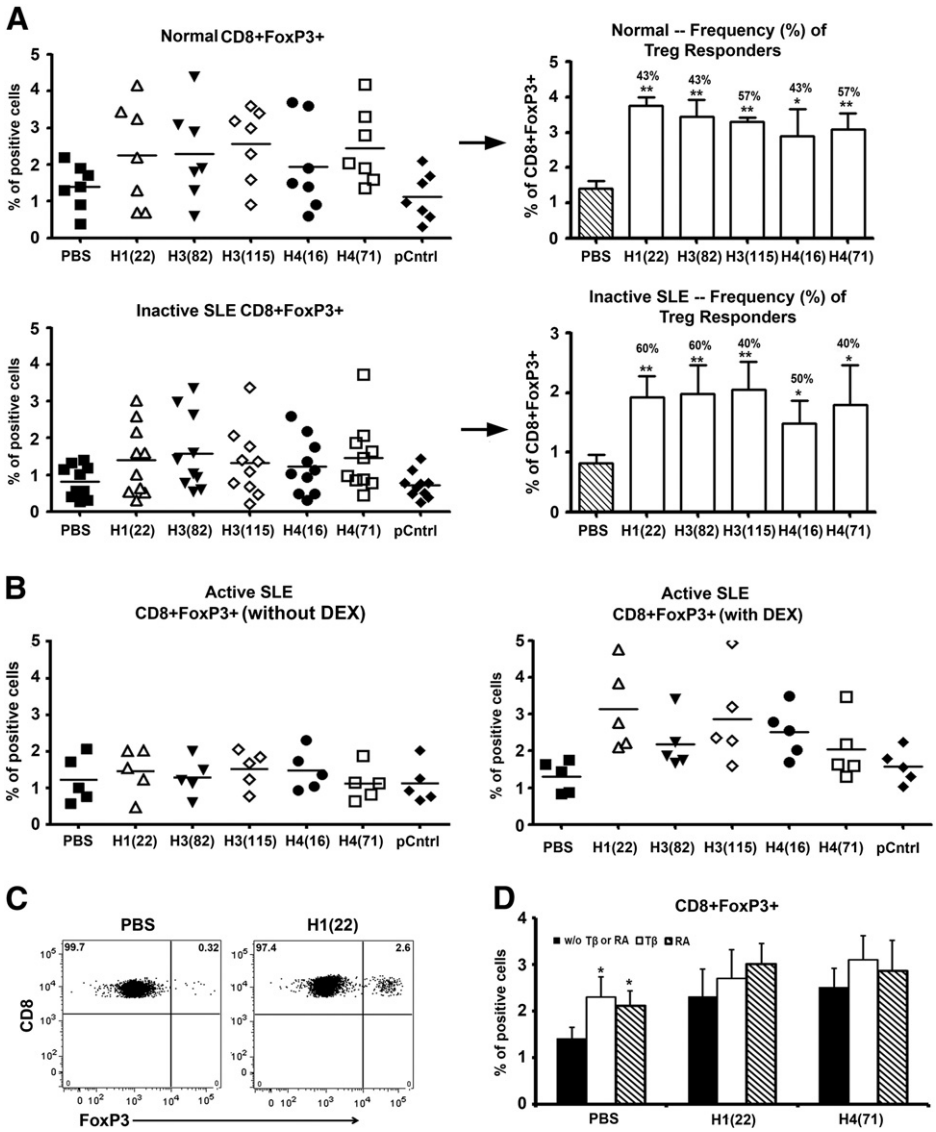


Figure 2 Durable induction of CD8$^+$FoxP3$^+$ cells in vitro by low-dose histone peptides. (A) Fresh PBMC samples derived from healthy subjects and inactive SLE patients were cultured as described in Fig. 1A and stained for CD8 and FoxP3. Y-axes show % of FoxP3 positive cells among viable T cells gated for being CD8$^+$. pCntrl = control peptide A; horizontal lines indicate the mean. Similar to the right panels in Fig. 1A and as explained in the corresponding legend of Fig. 1A, the right panels here (next to →) show the degree of CD8$^+$FoxP3$^+$ responses (bars = mean ± SD), except for the bar for PBS, and the frequency (% numbers above bars) of positive CD8 Treg responders for each peptide. $^{**}p < 0.01$, $^{*}p < 0.05$. n = 5 to 10. (B) Fresh PBMCs from active SLE patients were cultured with histone peptide epitopes and IL2, in the presence or absence of dexamethasone (DEX) for 7 days, and then stained for CD8 and FoxP3. (C) Representative Dot Plots show induction of CD8$^+$FoxP3$^+$ T cells in PBMC after culturing with the histone peptide epitope $H1_{22-42}$ for 7 days. Panel (D) Shows the effects of TGF-β (Tβ) or retinoic acid (RA) on induction of CD8$^+$FoxP3$^+$ Treg cells by two of the peptide epitopes, n = 3, $^{*}p < 0.05$.

(Figs. 1D and 2D, and data not shown). RA or TGFβ could increase the baseline for % of CD4 and CD8 Tregs without added peptides (PBS only), but could not significantly increase % Tregs induced by the peptide epitopes any further (Figs. 1D & 2D). Similarly, Rapamycin (RAPA) could increase the background of FoxP3 expression in T cells (with PBS only), but could not enhance histone peptide induced FoxP3$^+$ expression in T cells. Anti-IL6 neutralizing antibody also could not enhance histone peptide induced FoxP3$^+$ T cell expression any further, although it did by itself markedly increase FoxP3 and CD25 expression in CD4 and CD8 T cells in the 7-day PBMC cultures from normal and inactive lupus subjects (data not shown).

3.3. Low doses of histone peptides induced LAP expression on CD4 and CD8 T cells in vitro

In lupus-prone mice, the histone peptides induce Tregs that mediate disease suppression by TGFβ [22,23], and such remission inducing Treg can express latency-associated

peptide (LAP) on their cell surface and other TGFβ dependent markers, such as CD103 and CD39 [19]. Therefore, we added the histone peptides into the PBMCs from healthy subjects and patients with inactive SLE, and cultured the cells for one day or three days before staining for T cell expression of LAP, CD103 and CD39. After one day of culture both $CD4^+CD25^+$ T cells and $CD8^+$ T cells expressed markedly higher levels of TGFβ-LAP when cultured with histone peptides as compared to PBS (Fig. 3A), but CD103 (Fig. 3B) and CD39 expression did not increase significantly (data not shown).

3.4. Low doses of peptide epitopes induced Treg cells through TGFβ/ALK-5/pSmad 2 and 3 signaling pathway

Because the histone peptides induced high level LAP expression on CD4CD25 and CD8 T cells, we tested whether the epitopes induced Tregs through TGFβ/ALK-5/pSmad 2 and 3 signaling pathway. TGF-β is secreted in a latent form, which first needs to be activated by proteases or thrombospondin before it can bind to its specific type I and type II serine/threonine kinase receptors. Most cell types contain a TGFβ type I receptor active receptor-like kinase 5 (ALK5), which propagates the signal to the nucleus through phosphorylation of Smad2 and Smad3 proteins. Therefore, we assessed the effect of blocking TGFβ/ALK5/pSmad2 and 3 signaling by an ALK5 inhibitor (SD-431542). The PBMCs from active or inactive SLE patients were cultured with or without low-dose histone peptides in the presence or absence of ALK5 inhibitor SD-431542 (5 μM) for 7 days, and then $CD4^+CD25^{hi}FOXP3$ expression was analyzed. The ALK5 inhibitor markedly diminished the inducing effect of low-dose histone peptides for $CD4^+CD25^{hi}FOXP3^+$ cells (Fig. 4A). We also measured phosphorylated Smad2 and Smad3 protein expression on $CD4^+CD25^+$ cells and $CD8^+$ T cells after SLE PBMCs were cultured with low-dose

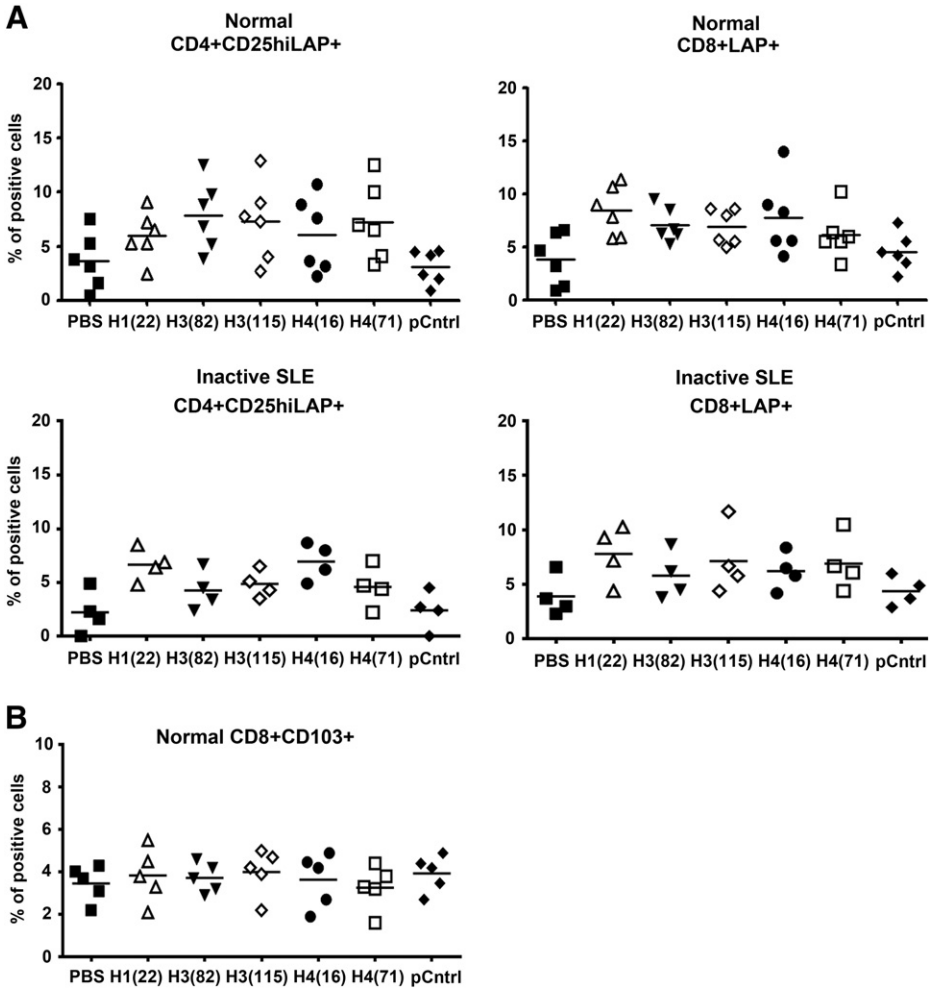


Figure 3 Low-dose histone peptides induce LAP expression on CD4 and CD8 T cells in vitro. (A) Fresh PBMC samples from healthy subjects (upper panels) and inactive SLE patients (lower panels) were cultured with peptide epitopes for 1 day before staining CD4, CD8, CD25 and LAP. Y-axes show % of LAP^+ cells among viable T cells gated for being $CD4^+CD25^{hi}$ or $CD8^+$, n = 4 and 6, the horizontal line = mean. *p* values for each histone peptide over controls (PBS or pCntrl) were <0.05 to <0.01; and PBS vs. pCntrl $p > 0.05$. (B) Fresh PBMC samples from healthy subjects were cultured with peptide epitopes for 3 days before staining for CD8 and CD103.

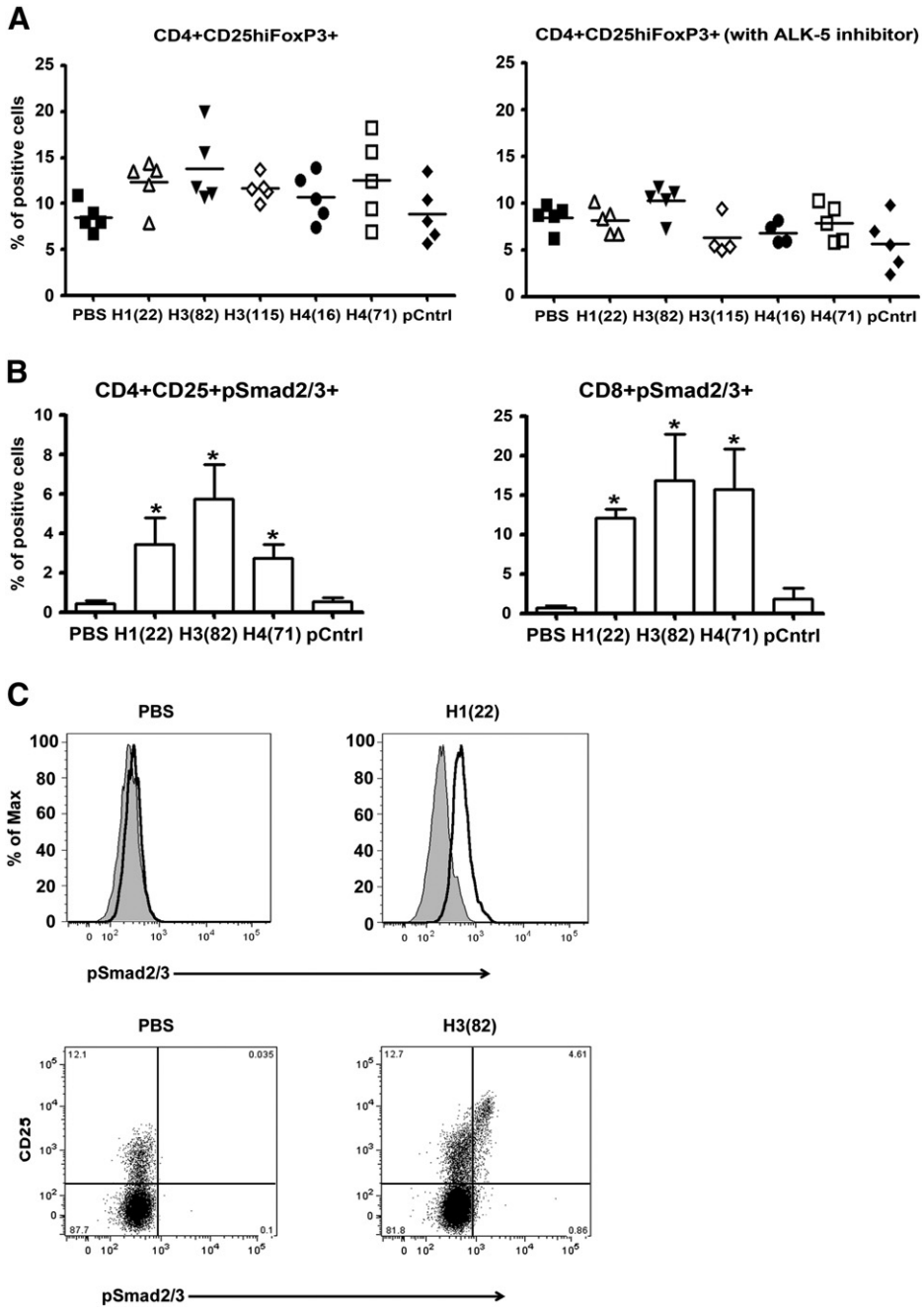


Figure 4 Low-dose peptide epitopes induce Treg cells through TGF/β-ALK-5/pSmad 2 and 3 signaling pathway. Active or inactive SLE PBMCs were cultured with peptide epitopes in the presence of IL2 or IL2 plus ALK-5 inhibitor SB-431542 for 7 days, followed by staining for CD4, CD25 and FoxP3. We also measured pSmad2/3 expression among viable CD4 or CD8 T cells under those conditions. (A) Y-axes show % of FoxP3 positive cells among viable T cells gated for being $CD4^+CD25^+$, n = 5, the horizontal line = mean. The *p* values for each peptide by ALK-5 inhibitor compared to no inhibitor were <0.05 to <0.01. (B) Y-axes show % of pSmad2/3 positive cells among viable T cells gated for being $CD4^+CD25^{hi}$, or $CD8^+$. Results from five experiments; and the bars = mean ± SD, $*p < 0.01$. (C) Upper 2 panels show representative histogram of pSmad2/3 positive cells (solid line) among $CD8^+$ cells (filled = isotype control). Lower 2 panels show representative Dot Plots of pSmad2/3 positive cells among $CD4^+CD25^+$ T cells.

histone peptides or the control peptide A for 7 days. We found that pSmad 2/3 expression in T cells was induced by the histone peptide epitopes, but PBS or control peptide A did not show such an effect (Fig. 4B). Moreover, Dot Plots showed that the pSmad 2/3 positive cells were within the $CD4^+CD25^{hi}$ subpopulation (Fig. 4C), suggesting that these cells belong to the peptide-induced Treg population.

3.5. Low dose of peptide epitopes individually or in a mixture (cocktail) inhibit the production of the pathogenic variety of autoantibodies by active lupus patient's PBMCs

Production of pathogenic autoantibodies, such as IgG autoantibodies to dsDNA, ssDNA and nucleosomes by B cells in vitro, requires T cell help and is detectable only in PBMCs from patients with active lupus [8]. Herein, we confirmed that the pathogenic IgG autoantibodies were detectable in PBMC cultures from patients with active lupus, but not inactive lupus. We then measured the suppressive function of low-dose single histone peptide on the production of pathogenic IgG autoantibodies by PBMCs from active lupus patients. Individual peptide showed significant suppressive effects on autoantibody production in PBMCs from active lupus patients (Fig. 5A). The five peptide epitopes tested individually suppressed (mean ± SEM of percent inhibition) IgG anti-dsDNA by 32% to 70%, IgG anti-ssDNA production by 41% to 74%, and IgG antinucleosome autoantibodies by 30% to 67% while the control peptide A had much less suppressive effect (Fig. 5B). The single peptides (low-dose) were present in PBMC cultures throughout the 13-d culture period (see Materials and methods); and drugs received by the patients when the blood was obtained are shown in Table 1.

However, not every peptide showed the suppressive function on every patient at all times; $H1_{22-42}$ and $H3_{115-135}$, were the most consistent inhibitors. Therefore, we measured the suppressive function of mixtures or cocktail of the different peptide epitopes on autoantibody production and found that cocktails showed very uniform and potent suppressive function, especially peptide cocktail-1: H1(22) + H3(115) + H4(16) at 1.5 μM/peptide, and peptide cocktail-3: H1(22) + H3(82) + H4(71) at 1.5 μM/peptide. The potency of the peptide cocktails was as good as anti-IL6 neutralizing antibody in suppressing autoantibody production (Fig. 5C).

3.6. Nucleosomal histone peptide cocktails suppress the expression of type I IFN related genes in lupus PBMC

Type I IFNs are produced at a high level, predominantly by pDC stimulated by anti-nuclear autoantibodies complexed with nuclear antigens in lupus [10–15], and in mouse models of lupus low-dose therapy by the histone peptide epitopes target pDC to make them tolerogenic [23]. For these reasons, we first tested whether the histone peptide epitopes individually or peptide cocktails cultured with $CD14^+$ monocytes from normal human peripheral blood under myeloid DC maturation culture conditions (GMCSF and IL4) could inhibit or delay maturation of myeloid DC. We measured DC surface markers of maturation by flow cytometry (such as CD1c, CD80 and HLA-DA) and gene expression for ITGB6, ITGB8, IL-10, TAB-1, BAFF, THBS1, and IL6 by real-time PCR at different culture time points (24 and 48 h). The results did not show any significant change (data not shown). Then we explored whether the low doses of histone peptides individually or peptide cocktails inhibited type I IFN induced gene expression in PBMCs from lupus patients. At the same time we measured autoantibody levels in supernatants of peptide treated PBMCs from active lupus patients described above, we also harvested the cells and measured the expression of type I IFN stimulated genes (ISG) from the cultured cells. In total, we measured 12 ISG genes reported to be upregulated in lupus patients [10,11,15], and the results showed that peptide cocktails (especially peptide cocktail-2 consisting of H1(22) + H3(115) + H4(16), at 4 μM/peptide) could significantly reduce type I IFN induced gene expression, particularly CXCL10, MX1, OAS3, EPSTI1 and RASAD2 (Fig. 6A), however, other inflammation or immune regulation related gene expression, including ISG15, IFI44, LY6E, BAFF, HERC5, SOCS1 and SOCS3, did not show significant change (data not shown). Finally, the peptide cocktail-2 also markedly reduced expression of type I IFN genes themselves in TLR9 stimulated PBMCs from inactive lupus patients which were cultured for 5 days with TLR9-ligand CPG-containing oligonucleotide (ODN) 2216, and the suppressed type I IFN genes included INFα-1, INFα-2, INFα-4 and INF-β (Fig. 6B, upper panel), that are the major members of this family [38]. However, when the T and B cells were depleted from the whole PBMC before starting the 5-day cultures, the suppressive effect of peptide cocktail-2 on type I IFN gene expression disappeared completely (Fig. 6B lower panel). Because the peptide-induced Treg cells appear in significant numbers only after 5 days of culture, they could not be depleted at the onset of the cultures.

4. Discussion

Each of the histone peptide epitopes efficiently induced expansion of both $CD4^+$ and $CD8^+$ Treg in vitro in lupus patients' PBMC, similar to what they do in vivo in lupus-prone mice [22,23]. Treg induction by the peptides was evident in PBMC of normal subjects and inactive lupus patients, but in active patients, dexamethasone or hydroxychloroquine treatment of the cultures brought out the Treg inducing ability. Thus, the peptide epitopes could be most beneficial in maintaining patients in remission by inducing Treg after inflammation is suppressed. The peptides induced Treg even in the presence of patients' serum, and without assistance from exogenously added TGFβ, retinoic acid or other Treg enhancing agents. Peptide-induced Treg cells with $FoxP3^+$ phenotype steadily increased in the cultures from day 3 onwards for up to 11 days tested. Because FoxP3 expression can occur transiently on human T cell activation, it was reassuring that $FoxP3^+$ T cells were maintained throughout the test period of 11 days. Moreover, confirming that the Treg induced were real, TGF-β precursor LAP was also induced in both Treg populations by the epitopes. Furthermore, the tolerogenic signal generated by the epitopes in the iTreg cells required TGFβ signaling, as evidenced by phosphorylation of Smad-2 and 3. Blocking TGF-β signal by an ALK 5 inhibitor further confirmed that endogenous TGFβ signal was involved in generation and maintenance of the iTreg cells. In animal models these epitopes could induce a tolerogenic program by decreasing IL-6 and increasing TGF-β production by pDCs, which in turn induce Smad-3 phosphorylation (TGF-β signal) in target autoimmune T cells [23,39]. However, in the case of human lupus Treg cells induced here, Smad-2/3 phosphorylation was evident even after 7 days in the T cells, when most APCs could be dead in the culture, indicating a stabilized program of intrinsic and ongoing TGFβ signaling possibly by autocrine and paracrine TGFβ produced by the iTreg cells. It

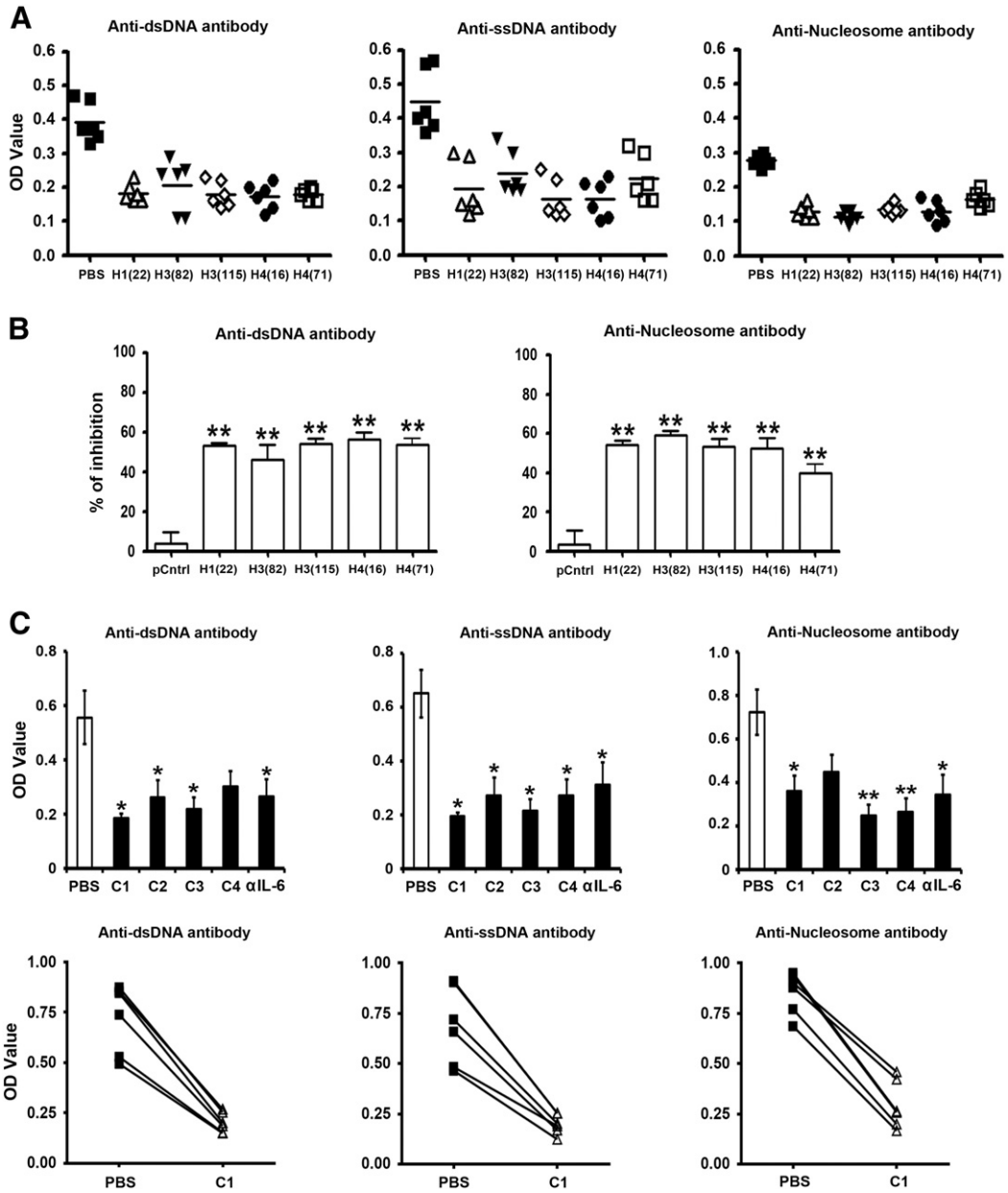


Figure 5 Low-dose peptide epitopes individually and as peptide cocktails mediate potent suppression of pathogenic autoantibody production by PBMC from active lupus patients. (A) The suppressive effect of single peptide epitope on the production of pathogenic autoantibodies. Y-axes show the absorbance at 405 nm. The horizontal line indicates the mean. (B) The data in "A" is shown as % of inhibition by each peptide as compared to control peptide A (pCntrl). (C) The upper panels show the suppressive effect of cocktails of peptide epitopes or a low dose of anti-IL6 antibody (600 ng/ml) on the production of pathogenic autoantibodies by PBMC in the culture. Peptide cocktails: C1 is H1(22) + H3(115) + H4(16) at1.5 μM/peptide. C2 is H1(22) + H3(115) + H4(16) at 4 μM/peptide. C3 is H1(22) + H3(82) + H4(71) at 1.5 μM/peptide. C4 is H1(22) + H3(82) + H3(115) + H4(16) + H4(71) at 1.0 μM/peptide. The bars = mean ± SEM,.The lower panels show the examples of inhibition of autoantibodies by cocktail-1(C1) in three independent experiments; Y-axes show the absorbance value at 405 nm. $^{*}p < 0.05$, $^{**}p < 0.01$.

was necessary for us to add IL-2 once to all the cultures including controls, at the beginning of the 7–11 day cultures for survival of the T cells. IL-2 could have contributed to the peptide induced Treg generation as in other systems [40,41]. However, IL-2 was also added to the cultures with control peptide or PBS, which did not show any Treg inducing effect. IL-2 production is impaired in lupus [42,43], and low dose IL-2 administration may facilitate $CD4^{+}CD25^{+}$ Treg homeostasis in chronic GVHD [44]. However, the histone peptide epitopes administered by themselves, without exogenous IL-2, can induce expansion of potent Treg in vivo, blocking lupus disease in three different mouse models of spontaneous SLE, namely, SNF1, B/WF1 and MRL-*lpr* [21–24,34].

In other approaches, a synthetic peptide (pConsensus) related to V_H region of anti-DNA antibodies, or a human Ig VH4-family CDR1 peptide could suppress lupus in mice and

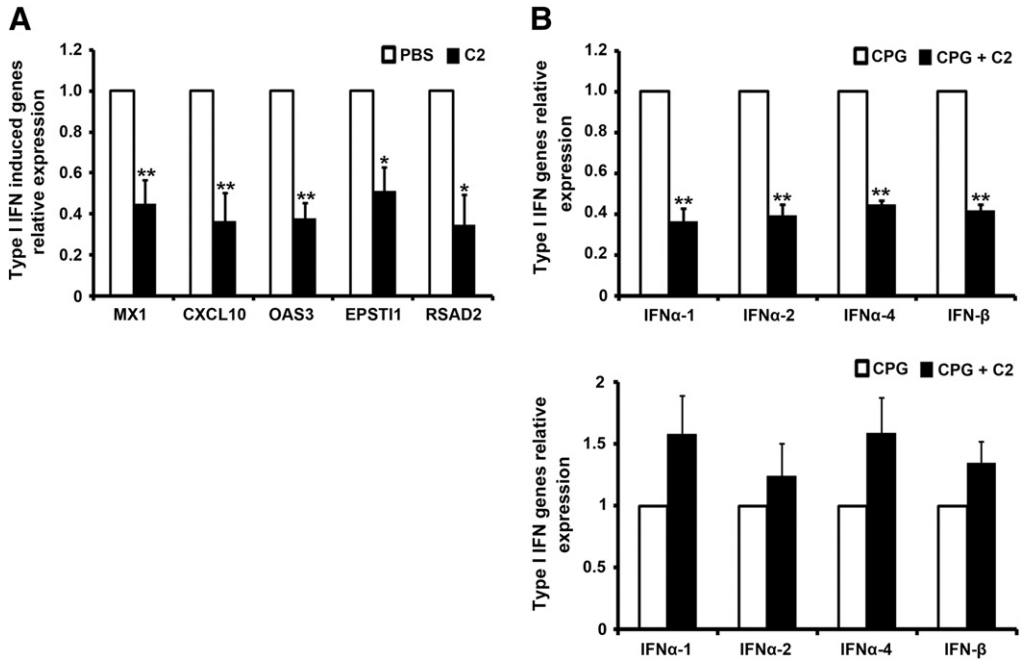


Figure 6 Histone peptide cocktails suppress expression of type I IFN genes and IFN-induced genes in lupus PBMC. (A) PBMCs from active SLE patients were treated with or without peptide cocktail-2 (C2) for 13 days before mRNA of type I IFN induced genes was quantified by qPCR. The bars represent the mean ± SEM from five individual experiments. (B) The upper panel shows results with whole PBMC from inactive SLE patients who were taking very low doses of steroids. The PBMCs were cultured for five days in the presence or absence of peptide cocktail-2 and then stimulated with TLR9 ligand CPG-containing oligonucleotide (ODN) 2216 for 4 h before type I IFN gene mRNA level was quantified by qPCR. The lower panel shows the qPCR results of the same experiments as done in upper panel but T cells were depleted from the PBMC before cell culture. The results show mean ± SEM, from four individual experiments. $^{*}p < 0.01$, $^{**}p < 0.001$.

induce iTreg cells [27–29,45]. Induction of $CD4^{+}25^{+}$ Treg in vitro has also been demonstrated with other peptide antigens in low doses [46,47]. Importantly, in vivo, antigen specific T cell receptor stimulation is required to further sustain functionally stable Treg [48]. Therefore, even adoptively transferred Treg cells generated first in vitro may require periodic administration of peptide epitope vaccine to maintain and further generate new antigen specific Treg recruits by tolerance spreading [20,22,23,31].

The $CD4^{+}CD25^{+}$ Treg generated by the histone peptide epitopes mediate their suppression by TGFβ [22,23], resembling the Treg induced by mucosal tolerance [49], and as in lupus-prone mice, these iTreg will be beneficial in lupus patients who suffer a deficiency in $CD4^{+}CD25^{+}$ Treg [19,50].

On the other hand, very little is known about the $CD8^{+}$ iTreg generated by the peptides in humans, although studies in animal models, indicate their key role in preventing, and suppressing established disease [19,22,28,30,31,51]. The peptide cocktail vaccine provides an opportunity to study the unusual iTreg cells in human lupus. Remarkably, HSCT induced remission in refractory lupus patients is dependent on the same type of potent CD8 Tregs generated by nucleosomal peptide therapy in lupus-prone mice [19], which act by producing TGFβ, and are *distinct* from other CD8 Treg cells that act by contact cytotoxicity [52–54]. Overall, the Treg induced by the histone peptide epitopes are both autoantigen specific and polyclonal Treg [19,22,23], probably because of the promiscuous nature of lupus autoantigenic epitopes [55], and also tolerance spreading by tolerogenic APC and Treg cells themselves that secrete TGFβ [19,22,23]. However, suppression by the Treg is preferentially directed against lupus autoimmune response overall, as immune response to foreign antigens is not compromised [22,23]. Herein, in humans, the peptide epitopes significantly increased $CD4^{+}CD45RA^{+}FoxP3^{low}$, but did not increase $CD4^{+}CD45RA^{-}FoxP3^{hi}$ cells (Fig. 1C), indicating that most of the Treg were newly induced by the peptide epitopes.

Each of the histone peptide epitopes also inhibited pathogenic IgG autoantibody production in vitro by the PBMC of active lupus patients, without any addition of steroids or exogenous TGFβ. These patients' cells were producing relatively high levels of autoantibodies to the nuclear antigens to begin with, so that marked inhibition by the peptides (up to 74%) in vitro could be evident. Cocktails of the epitopes in different combinations were even more efficient in uniformly suppressing pathogenic autoantibody production; especially the cocktail C1 inhibited up to 90%. The inhibition of autoantibody production by the epitope cocktails was even superior to anti-IL6 antibody. This potency of the peptide epitopes is very encouraging, because anti-IL6 therapy, although targeting a critical pathogenic cytokines in lupus has serious side effects [56]. The iTreg generated by the peptide epitopes could have suppressed autoimmune Th cells and APC [23,29,57]. However, induction of conventional Treg by the peptides in *active* lupus PBMC was relatively less efficient in contrast to that in inactive lupus patients or healthy subjects, yet surprisingly the epitopes caused a marked inhibition of autoantibody production in *active* lupus

patients' PBMC. These results indicate that lupus B cells that also recognize the epitopes could have been directly tolerized by the peptides [18,58], in addition to their direct tolerogenic effect on autoantigen presenting pDC, inducing them to produce endogenous TGFβ [23]. Inhibition of autoantibody production by the peptide epitopes was real and not due to binding up of the autoantibodies in culture supernatants by the peptides via nuclear antigens in the autoantibody combining sites, because addition of the peptides to serial dilutions of active lupus patient's plasma did not make a difference in IgG anti-nuclear autoantibody titer measurements.

The peptides also suppressed IFNα gene expression by lupus PBMC, which was dependent on T cells. This result was probably through generation of iTreg by the epitopes, which then suppressed IFN producing APC by TGFβ. In addition, the peptide epitopes could have a direct tolerogenic effect on pDC in the early stages of the cultures to facilitate the induction of iTreg, as found in mouse models [23].

4.1. Concluding remarks

Developing tolerance therapy for SLE that specifically targets pathogenic autoimmune cells by utilizing the major nucleosome-derived peptide epitopes would have the following advantages: a) the peptides are unaltered, naturally occurring, ubiquitous peptide ligands (UPL), expressed in the thymus and bone marrow during ontogeny of the immune system, and therefore, unlike artificially altered peptide ligands or APLs [59], they are not associated with anaphylactic/allergic reactions [20,22,23,60]; b) they are effective at very low doses, and by subcutaneous or intranasal administration in animal models of lupus; c) they generate long-lasting, antigen-specific iTreg that suppress disease pathology; d) they induce cross-reactive, "tolerance spreading" or linked tolerance to other pathogenic T and B cell autoepitopes of lupus, but not to exogenous antigens; and e) they are recognized by autoimmune T and B cells of all lupus *patients* tested irrespective of their HLA type [2,17–20,55]. The histone peptide epitope therapy might be most suitable for restoring immunoregulation and maintaining lupus patients in remission after inflammation is suppressed by more toxic or global immunosuppressive agents. Importantly, the peptide epitopes are effective even when the autoimmune disease is already established [20,22,23].

Conflict of interest statement

This work did not receive any support from commercial sources. The histone peptide epitope sequences have been published by us repeatedly [2,16–18], and they are available in databases open to the public, such as in:

http://www.iedb.org/list_page.php?list_type=tcell&measured_response=Positive&total_rows=32&queryType=true
http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

Many other investigators have published their work independently in their own experimental systems using the peptide epitopes we have identified [21,24,34]. The peptide epitope sequences have been patented, but have not been licensed to any commercial entities. The NIH and Northwestern University have the main rights to the patented discoveries. We have published the sequences of the peptides for open access to everyone.

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