Low-Dose Peptide Tolerance Therapy of Lupus Generates Plasmacytoid Dendritic Cells That Cause Expansion of Autoantigen-Specific Regulatory T Cells and Contraction of Inflammatory Th17 Cells

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Low-Dose Peptide Tolerance Therapy of Lupus Generates Plasmacytoid Dendritic Cells That Cause Expansion of Autoantigen-Specific Regulatory T Cells and Contraction of Inflammatory Th17 Cells¹,²

Hee-Kap Kang, Michael Liu, and Syamal K. Datta³

Subnanomolar doses of an unaltered, naturally occurring nucleosomal histone peptide epitope, H₄₇₁₋₉₄, when injected s.c. into lupus-prone mice, markedly prolong lifespan by generating CD4⁺₂₅⁺ and CD8⁺ regulatory T cells (Treg) producing TGF-β. The induced Treg cells suppress nuclear autoantigen-specific Th and B cells and block renal inflammation. Splenic dendritic cells (DC) captured the s.c.-injected H₄₇₁₋₉₄ peptide rapidly and expressed a tolerogenic phenotype. The DC of the tolerized animal, especially plasmacytoid DC, produced increased amounts of TGF-β, but diminished IL-6 on stimulation via the TLR-9 pathway by nucleosome autoantigen and other ligands; and those plasmacytoid DC blocked lupus autoimmune disease by simultaneously inducing autoantigen-specific Treg and suppressing inflammatory Th17 cells that infiltrated the kidneys of untreated lupus mice. Low-dose tolerance with H₄₇₁₋₉₄ was effective even though the lupus immune system is spontaneously preprimed to react to the autoepitope. Thus, H₄₇₁₋₉₄ peptide tolerance therapy that preferentially targets pathogenic autoimmune cells could spare lupus patients from chronically receiving toxic agents or global immunosuppressants and maintain remission by restoring autoantigen-specific Treg cells. The Journal of Immunology, 2007, 178: 7849–7858.

Tolerance therapy for systemic lupus erythematosus using autoantigenic peptides that specifically target pathogenic autoimmune cells is a highly desirable goal (1–5). Due to intrinsic defects in lupus immune system, nucleosomes from apoptotic cells become major immunogens initiating cognate interactions between autoimmune CD4⁺ Th cells and B cells leading to production of somatically mutated, class-switched autoantibodies that form pathogenic immune complexes with diverse nuclear Ags (6–11). Certain peptides in nucleosomal histones are dominant autoepitopes and spontaneous priming to these occurs in preclinical lupus (12, 13). These epitopes are cross-reactively recognized by autoimmune Th cells, as well as B cells of lupus, and they can be promiscuously presented in the context of diverse MHC class II (MHC II)⁴ alleles, like “universal epitopes” (2, 12–14). These unaltered, native peptide epitopes halt progression of lupus nephritis upon tolerization in high-dose soluble form and importantly for human therapy, the peptides are also effective in delaying/preventing lupus nephritis in subnanomolar doses (~0.37 nM or 1 μg), administered s.c. to lupus-prone SNF₁ mice (2, 3, 15). This dose is 300- to 1000-fold less than peptides from other nucleoproteins or unrelated peptides from Ig V regions (CDR) that are being tried as therapeutic agents (4, 5, 16, 17).

Among nucleosomal histone epitopes, H₄₇₁₋₉₄ is highly potent in low-dose tolerance therapy, because it cross-reactively suppresses autoimmunity to other pathogenic epitopes and whole nucleosomes (2, 3). Moreover, H₄₇₁₋₉₄ also can suppress lupus via nasal tolerance (18). Importantly, H₄₇₁₋₉₄ also binds strongly to common HLA-DR alleles. T cells reactive to this epitope can be detected in almost all lupus patients, as well as SNF₁ and BWF₁ mice (2, 12, 13, 19, 20).

The mechanism of the decades-old phenomenon of low-dose tolerance or “immunologic paralysis” (21–23) was unknown and even recent mechanistic studies (24) have dealt with tolerance in naïve animals to foreign Ags. In lupus, we are studying low-dose tolerance in an immune system that is already primed spontaneously to high doses of the same nucleosomal autoepitope that is ubiquitous. Moreover, numerous traits for disease susceptibility were intact in the immune system of the spontaneous systemic lupus erythematosus model studied here. The complex cellular interactions were not curtailed or modified from birth by any transgenic or knockout approaches, nor have we added any exogenous TGF-β to help augment tolerance (24) because that might be detrimental in the presence of excessive IL-6 production in lupus (25, 26). Indeed, we were surprised that therapeutic tolerance with H₄₇₁₋₉₄ peptide could be achieved in the face of complex lupus traits like intrinsic hyperactivity of B cells, T cells, and dendritic cells (DC), particularly plasmacytoid DC (pDC) making excessive IFN-α (27–29). Because low-dose tolerance with H₄₇₁₋₉₄ peptide induces CD8⁻, and CD4⁺CD₂₅⁺ adaptive regulatory T (Treg)
cell subsets that produce TGF-β, and is highly potent in suppressing lupus nephritis (3), herein, we investigated the mechanism of Treg generation.

Materials and Methods

Mice

New Zealand Black and SWR mice were purchased from The Jackson Laboratory. Lupus-prone SNF1 hybrids were bred and females were used, as approved by the animal care and use committee.

Peptides

All peptides were synthesized by F-moc chemistry and their purity was checked by amino acid analysis by the manufacturer (Chiron Mimotopes).

Tolerance induction with very low doses of peptides

For low-dose tolerance studies, serologically autoimmune, but prenephritic, 12-wk-old SNF1 females (nine mice per group) were injected s.c. with H471–94, three times, at 2-wk intervals. One week later, the recipient mice were immunized with a lupus-accelerating nucleosomal peptide, H1/22–43, in CFA. The animals were monitored for nephritis, survival, and IgG autoantibodies for 5 mo after final adoptive transfer.

To test the immunological consequences of transferring DCs or B cells in recipient mice, another batch of 3-mo-old SNF1 mice (five per group) were treated as above with or without immunization with H1/22–43 in CFA. Ten days after the third transfer (1 x 105 cells/mouse for each transfer), these short-term batches of mice were sacrificed for analysis of autoimmune T and B cells and Treg cells using ELISPOT and/or ELISA for cytokines and IgG autoantibodies.

Immunohistochemistry

One-half of each kidney from tolerant or control mice was fixed in 10% formalin and paraffin embedded. Paraffin sections were used for immunohistochemical analysis of Th17 infiltration, as described (3).

ELISPOT assay

ELISPOT assay plates (Cellular Technology) were coated with capture Abs against IFN-γ or IL-17 (BD Pharmingen) in PBS at 4°C overnight. Splenic T cells (1 x 104) from treated mice were cultured with irradiated (3000 rad) splenic B cells, macrophages, and DC from 1-mo-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 Ab biotin and subsequent alkaline phosphatase-conjugated streptavidin. Cytokine-expressing cells were detected by immunoslot scanning and analysis (Cellular Technology).

Suppression assay

Ten days after final (third) adoptive transfer of H471–94-tolerized or PBS-treated pDC or non-pDC, CD4+CD25+ and CD8+ Treg cells (1 x 105) were isolated from recipient mice and the ability of Treg cells to directly inhibit IFN-γ responses of unmanipulated SNF1 lupus T cells to nucleosomes presented by APC were compared in ELISPOT assays. The ratios of Treg-lupus Th cells were 1:1, 1:2, 1:4, 1:10, and 1:40 (3).

Cytokine ELISA

Splenocytes (1 x 106), T cell-depleted APC (5 x 105) plus T cells (1 x 104) from DC-recipient or unmanipulated SNF1 mice, were stimulated with H471–94 peptide, nucleosomes, or anti-CD3 (1 μg/ml). Culture supernatants were collected after 90 h for TGF-β1, or 72 h for IFN-α, IL-6, IL-10, IL-12, IL-23, and IL-17. Amounts of IL-6, IL-10, and IL-12 were measured by the OptEIA ELISA set (BD Pharmingen). The amount of IFN-α was measured by using an ELISA kit (PBL Biomedical Laboratory). For TGF-β1, samples were acidified by addition of HCl at 20 mM for 15 min and neutralized by NaOH and then the amount of TGF-β1 was measured by TGF-β1 Emax ImmunoAssay System (Promega). Amounts of IL-17 and IL-23 were measured by IL-17 and IL-23 ELISA Ready-sets (e Bioscience).

Flow cytometry

For analyzing surface markers on DC, whole splenocyte populations were prepared by collagenase-DNase digestion followed by washes in EDTA-containing buffer to prevent clumping as described (3). To avoid unnecessary manipulations, further purifications were not done, but CD11c-FITC- and B220-allophycocyanin-positive cells were gated and analyzed after three-color staining of splenocytes. FITC-labeled anti-CD11c Ab, PE-labeled anti-MHCII (M5/114.15.2), anti-CD83, anti-CD80, anti-CD86, anti-CD40, allophycocyanin-labeled anti-B220 Ab, and isotype controls were obtained from BD Biosciences; PE-labeled anti-CD205 Ab was obtained from Cedarlane Laboratories. PE-conjugated Ab to TGF-β from IQ products (Biotest Diagnostic) was used for intracellular staining. For intracellular staining of Th17 cells, splenocytes from H471–94-tolerized or PBS-treated SNF1 mice were cultured for 48 h with IL-6 and/or nucleosomes and then Golgi stop (e Bioscience) was added to cultures 12 h before cell staining. We also stimulated splenocytes from H471–94-tolerized or PBS-treated SNF1 mice with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μM; Calbiochem) for 4–5 h and then Golgi stop was added during last 2 h, as described (25). Cells were then stained with CD4-FITC, fixed, and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), and were then stained with IL-17-PE (BD Biosciences).

CFSE-based cell proliferation assays

T cells from H471–94-tolerized or PBS-treated SNF1 mice were labeled with CFSE using the Vybrant Cell Tracer kit from Molecular Probes following
the manufacturer’s protocol. CFSE-labeled T cells (1 × 10^6) were cocultured with H471–94-tolerized DC or PBS-treated DCs (2.5 × 10^5) in the presence or absence of Ag in criss-cross combinations for 72 h, and then stained with PerCP Cy5.5-labeled anti-CD4, allophycocyanin-labeled CD25 (BD Biosciences), and PE-labeled foxp3 Abs (eBioscience), or allophycocyanin-labeled CD8 (BD Biosciences) and PE-labeled TGF-β Abs (IQ Products). We performed flow cytometry to compare proliferation by gating on CD4<sup>+</sup>CD25<sup>+</sup>foxp3<sup>+</sup> cells or on CD8<sup>+</sup>TGF-β<sup>+</sup> cells among CFSE-labeled cells by using Cyan ADP (DakoCytomation) with Summit software and FACS express 3 software (De Novo Software).
Real-time RT-PCR

Ten days after the third injection of H471-94 (1 μg) in a low-dose tolerance regimen, we measured cytokine and indoleamine 2,3 dioxygenase (IDO) mRNAs by real-time PCR in whole DCs, or subsets (pDCs and non-pDCs) from H471-94-tolerized or PBS control mice without further stimulation. To measure expression of cytokine mRNAs, total RNAs from DC or DC subsets from low-dose peptide-tolerized or PBS control mice were isolated by the RNeasy kit (Qiagen) and then cDNA was synthesized using a high-capacity cDNA archive kit (Applied Biosystems). Expressions of IFN-α, IL-6, IL-10, IL-12, TGF-β, and IDO mRNA were measured using Assays-on-Demand gene expression products and TaqMan Universal PCR master mix (Applied Biosystems). GAPDH was used as an endogenous reference. We analyzed data as described (3) using ABI 7700 Sequence Detection system software (Applied Biosystems).

Statistical analysis

The log-rank and the Student two-tailed t tests were used. Results are expressed as the mean ± SEM, unless noted otherwise.

Results

Fate of injected nucleosomal histone peptide

Because s.c. injection of low-dose peptide generated potent Treg in spleen (3), we investigated which APC were involved. Within 24 h of s.c. injection of H471-94, both splenic DCs and B cells stimulated a highly sensitive, H471-94-specific T cell hybridoma without further addition of the exogenous peptide (Fig. 1A). The DCs presented captured peptides more efficiently. The APCs of control PBS-treated animals stimulated weakly, probably presenting endogenously acquired autoantigens (12, 30). DC and B cells from the draining lymph nodes did not stimulate the cognate T cell hybridoma (data not shown).

The DCs, but not B cells, of the H471-94-tolerized animals blocked lupus acceleration

Adoptive transfer of the splenic DCs of H471-94-tolerized animals delayed the onset of accelerated lupus nephritis in H1-22-42-immunized SNF1 mice (Fig. 1B) and prolonged survival (Fig. 1C). In the nephritis acceleration assay, young SNF1 mice immunized with another nucleosomal epitope, H1-22-42 in advaut develop severe nephritis and produce high level of autoantibodies much more rapidly, as described (3, 30). Between 8 and 10 wk after final (third) adoptive transfer of H471-94-tolerized DC, only 20% of recipient mice had severe nephritis, whereas 80% of mice receiving PBS-treated control DC had severe nephritis (p < 0.01). Between 16 to 20 wk after final transfer of H471-94-tolerized DCs, 100% of recipient mice had survived, whereas 50% of mice receiving control DCs were dead (p < 0.01). Transfer of H471-94-tolerized animal B cells had no significant effect (Fig. 1, B and C).

The DC and subsets from H471-94-tolerized animals diminished IgG autoantibodies and T cell responses to nucleosomes

Adoptive transfer of H471-94-tolerized DCs reduced the levels of the pathogenic (C′ fixing, inflammatory FcγR binding) subclass of IgG2a autoantibodies to dsDNA, ssDNA, nucleosomes, and histones by 42, 26, 54, and 64%, respectively (p < 0.001–<0.05, Fig. 1D), but transfer of the B cells of the H471-94-tolerized animals did not, except for histones. Adoptive transfer of the DCs of H471-94-tolerized animals diminished recipient T cell responses to nucleosomes up to 70%, as compared with control recipients (Fig. 1E, p < 0.01), whereas transfer of H471-94-tolerized B cells did not (Fig. 1E, p > 0.05).

We further isolated subsets from H471-94 tolerized animal’s DCs into plasmacytoid DC (pDC) and non-pDC for adoptive transfer. Ten days after the third transfer, we analyzed the levels of pathogenic IgG autoantibodies in serum and IFN-γ response to nucleosomal autoantigen by T cells of recipient mice. Both subsets of H471-94 tolerized DCs reduced pathogenic IgG2a and IgG2b subclasses of autoantibodies in recipients (Fig. 2A, p < 0.02). Adoptive transfer of H471-94-tolerized pDC, but not non-pDC, markedly reduced the levels of pathogenic IgG subclasses of autoantibodies in recipient mice, as compared with controls. Percent reduction in autoantibody to dsDNA, ssDNA, nucleosomes, and histone, respectively, were as follows: IgG2a autoantibodies by H471-94-tolerized pDC: 49, 34, 36, and 93%; IgG2b by H471-94-tolerized pDC: 57, 75, 68, and 98%; IgG2a by H471-94-tolerized non-pDC: 60, 51, 61, and 98%; IgG2b by H471-94-tolerized non-pDC: 55, 65, 26, and 94% (n = 5). B, Adoptive transfer of H471-94-tolerized pDCs, but not non-pDC, decreased IFN-γ responses to nucleosomes by lupus T cells in ELISPOT, as compared with that of control-treated pDCs. Splenic T cells from SNF1 recipients of H471-94-tolerized or PBS-treated animal DC subsets were challenged with nucleosomes in various concentrations in vitro. Baseline of IFN-γ responses was 5 ± 3 spots/1 × 10^6 T cells. Data from three experiments (n = 5). *, p < 0.001; x, p < 0.02; +, p < 0.05 (t test).

Transfer of the pDCs of H471-94-tolerized animals caused increased TGF-β and decreased IL-17 production by recipient T cells

We observed significantly increased amount of TGF-β in culture of T cells in splenocytes from recipients of H471-94-tolerized pDCs...
Unmanipulated lupus-prone SNF1 mice had markedly increased Th17 response to nucleosome autoantigens, as compared with non-autoimmune, SWR, or C57BL/6 mice (Fig. 3A), and low-dose tolerance with H471–94 (1 μg) markedly reduced their IL-17 production in response to nucleosomes by ELISPOT (Fig. 3B). Flow cytometry also showed significantly reduced Th17 cells upon autoantigen stimulation in tolerized SNF1 mice (Table I). We could not detect any significant Th17 cells in SNF1 mice without autoantigen stimulation, even upon ionomycin and PMA stimulation (data not shown).

We observed infiltration of Th17 cells in kidneys from control-treated mice by immunohistochemistry, whereas no such infiltrates in kidneys from H471–94-tolerized mice, indicating that low-dose tolerance inhibited expansion and migration of activated Th17 cells to target organs (Fig. 3F).

**Low-dose tolerance with H471–94 increases TGF-β, but decreases IL-6 production by DCs**

Because adoptive transfer of tolerized pDC caused TGF-β production by T cells of recipients, we analyzed the DCs themselves. Without further stimulation, we observed significant increases of TGF-β and IL-10 mRNA in whole DCs from H471–94-tolerized mice, as compared with controls (Fig. 4A), but no significant change in IDO mRNA (p > 0.05). In DC subsets, TGF-β mRNA in H471–94-tolerized pDCs was 4-fold higher than PBS control pDCs (Fig. 4A, p < 0.001), but no significant differences were seen in non-pDCs (Fig. 4A).

IL-10 production by whole splenocytes or by T cell-depleted splenocytes from recipient mice (data not shown). C, Splenocytes from recipient mice of H471–94-tolerized pDC produced markedly decreased IL-17 in response to autoantigen, but transfer of non-pDC showed no significant effect. D, Unmanipulated, lupus-prone SNF1 mice produced markedly increased amounts of IL-17 in response to nucleosomes, in contrast to normal strains, SWR, and C57BL/6. E, Splenocytes (T cells) from H471–94-tolerized SNF1 mice produced markedly reduced amount of IL-17 upon nucleosome stimulation in vitro, as compared with those of control-treated mice. F, Representative immunohistochemistry (original magnification, ×200). Marked perivascular, interstitial, and glomerular infiltration of Th17 cells were detected only in kidneys of control-treated mice (left panel). A–E, Results are expressed in mean ± SEM from three experiments (five mice per group) and (F) is shown as representative (five mice/group). *p < 0.001; †p < 0.02; ‡p < 0.05 (t test).
**FIGURE 4.** Low-dose H471–94 tolerance increases TGF-β mRNA expression in DCs, especially pDC. A, TGF-β mRNA (real-time PCR) was markedly increased in the pDC of the H471–94-tolerized animal (H4 71-94 pDC) as compared with the pDC of the PBS-treated animal (PBS pDC). Low-dose peptide tolerance did not change relative expression of TGF-β in non-pDC. Therefore, the increase in relative mRNA expression of TGF-β in whole DC (H4 71-94 DC) after low-dose tolerance was probably contributed by the pDC subset. B, DCs from H471–94-tolerized mice produced markedly increased amount of TGF-β on stimulation with poly (I:C), CpG DNA, and nucleosomes, but not with LPS. Amounts of TGF-β in culture supernatants of DCs were measured by ELISA. C, DCs from H471–94-tolerized mice produced markedly decreased amount of IL-6 measured by ELISA. D, Left, Increased production of TGF-β by H471–94-tolerized DC on stimulation with nucleosomes (30 μg/ml) is mediated by TLR9 in endosome-lysosome compartment. TLR9 inhibitor abrogated the increased amount of TGF-β production by DC from animals receiving low-dose tolerance regimen. Right, Decreased production of IL-6 by H471–94-tolerized DC on stimulation with nucleosomes is mediated by TLR9. TLR9 inhibitor abrogated the decrease in IL-6 production by DC in low-dose tolerance regimen. Data from three experiments (n = 5). *, p < 0.001; **, p < 0.01; +, p < 0.05 (t test).
Isolated pDC subsets die overnight in culture unlike in vivo adoptive transfer experiments or assays done with them immediately ex vivo. Therefore, any experiment described below that required culturing for several days could be done only with the whole DC population. Nevertheless, the results in Figs. 2B, 3A, 4A, and 5A show that the tolerogenic effects were mainly due to pDC.

The low-dose tolerance regimen with H471–94 also increased production of TGF-β by DCs on stimulation in vitro with poly (I:C), CpG, or nucleosomes, but not LPS (Fig. 4B, p < 0.001–0.01). In contrast, low-dose H471–94 tolerance markedly reduced IL-6 production by DCs stimulated by same agents (Fig. 4C, p < 0.001), as compared with control treatment. Amounts of IFN-γ, IL-12, and IL-23 were not significantly different in DC cultures of H471–94-tolerized versus control (data not shown).

TLR9 is involved in production of TGF-β from DCs in low-dose tolerance

As major lupus autoantigens, nucleosomes, and small nuclear ribonucleoprotein contain DNA and RNA, we analyzed whether TLRs were involved. We isolated DCs from low-dose H471–94-tolerized or PBS-treated animals, and stimulated DCs with nucleosomes in the presence of TLR9 inhibitor or TLR7 inhibitors. TLR9-specific inhibitor decreased TGF-β production by H471–94-tolerized DCs on stimulation with nucleosomes in vitro (Fig. 4D, p < 0.001–0.05), but TLR7 inhibitor did not (p > 0.05, data not shown).

In contrast, the TLR9 inhibitor increased IL-6 production by H471–94-tolerized DCs on stimulation with nucleosomes in vitro (Fig. 4D). The inhibitor was not toxic because the IL-6-enhancing effect was seen even at high doses that were required to inhibit large number of DCs (0.3–1 × 10^6 cells/well).

pDCs of H471–94-tolerized animals have a tolerogenic phenotype

As shown in Table II, among the pDC of H471–94-tolerized mice, the percentage of CD40^+ cells and their mean and median fluorescence intensity (MFI) were decreased by 73, 32, and 44%, respectively (p < 0.01, <0.05, <0.05), and the percent and MFI of CD80 were decreased by 29 and 20%, respectively (p < 0.05). Slight increases of CD86 and MHC II on H471–94-tolerized pDC were not significant (1.14- and 1.02-fold, respectively, p > 0.05), but MFI of CD86 was significantly reduced by 29% (p < 0.05). Percentage of CD83^+ cells (mature DC marker) was decreased by 18% and its mean fluorescence was decreased by 60% on H471–94-tolerized pDC (p < 0.05), but CD205, a tolerogenic DC receptor (33, 34) was not significantly changed (p > 0.05). Median fluorescence of PD-L1, which inhibits T cells (35), was increased on H471–94-tolerized pDC by 18%, as compared with control (p < 0.05). The changes in surface markers are not as striking as the functional changes in the pDC of tolerized animals, probably because the steps needed to isolate DCs may change their surface markers quickly to a relatively more activated phenotype.

Intracellular staining for TGF-β (data not shown in table) was consistent with results in Fig. 4. The whole DC of H471–94-tolerized animals had increased TGF-β^+ cells (45.20 ± 3%, p < 0.01), as compared with PBS-treated controls (27.35 ± 2), and the percentages of TGF-β^+ cells were also increased 1.25-fold in pDC and 1.81-fold in non-pDC subsets from low-dose H471–94-tolerized mice (p < 0.01). Furthermore, mean and median fluorescence of TGF-β^+ pDC by low-dose H471–94 tolerance were 2.5- and 1.96-fold higher than those of non-pDC (all p < 0.05).

Transfer of H471–94-tolerized pDC augmented suppressive function of both CD4^+ CD25^+ and CD8^+ Treg cells in recipients

Ten days after final (third) transfer of the DC subsets of low-dose H471–94-tolerized or PBS-treated animals, we isolated both CD4^+ CD25^+ and CD8^+ T cells from recipient mice and then cocultured the T cells with T cells from 5.5-mo-old unmanipulated Snf1 mice, in the presence of nucleosomes. As compared with CD4^+ CD25^+ and CD8^+ T cells from recipients of PBS-treated animal pDC (Fig. 5A, p < 0.001), both of those cell subsets from recipients of H471–94-tolerized pDC had up to 2.2-fold higher suppressing ability of the IFN-γ response to nucleosomes at 1:4 ratio (Treg cells-target lupus T cells, optimal ratio as titrated in Fig. 5B). However, adoptive transfer of H471–94-tolerized non-pDC did not increase suppressive function (Fig. 5A, p > 0.05).

Ex vivo expansion of autoantigen-specific CD4^+ CD25^+/Foxp3^+ Treg cells from low-dose tolerized mice by DCs

CFSE-labeled, whole T cells from low-dose H471–94-tolerized or PBS-treated Snf1 mice were cocultured for 72 h with DCs from low-dose H471–94-tolerized or PBS-treated Snf1 mice in the presence of H471–94 or nucleosomes and then stained for cell markers (see Materials and Methods). As shown in Fig. 5, C and D, CD4^+ CD25^+ Foxp3^+ T cells from low-dose H471–94-tolerized mice proliferated upon coculture with H471–94-tolerized or PBS-treated DCs in the presence of autoantigens (p < 0.001–0.05), however, CD4^+ CD25^+ Foxp3^+ T cells from PBS-treated control mice did not (p > 0.05). Both H471–94-tolerized DC and PBS-treated DC expanded relatively more CD4^+ CD25^+ Foxp3^+ Treg cells from low-dose H471–94-tolerized mice with addition of H471–94 peptide than with nucleosomes.

Under similar conditions, CD8^+ Treg cells were not expanded by DC with nucleosomes or H471–94, although the autoantigens

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**Table II. Surface markers of CD11c^+ and B220^−-gated (pDC) in spleen**

<table>
<thead>
<tr>
<th>Surface Markers</th>
<th>Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pDC of PBS-treated animals</td>
</tr>
<tr>
<td></td>
<td>% Positive</td>
</tr>
<tr>
<td>CD86</td>
<td>14.2 ± 4</td>
</tr>
<tr>
<td>CD40</td>
<td>43.8 ± 9</td>
</tr>
<tr>
<td>CD80</td>
<td>14.7 ± 2</td>
</tr>
<tr>
<td>MHC II</td>
<td>50.6 ± 1</td>
</tr>
<tr>
<td>CD205</td>
<td>10.9 ± 6</td>
</tr>
<tr>
<td>CD83</td>
<td>9.4 ± 6</td>
</tr>
<tr>
<td>PD-L1</td>
<td>60.09 ± 14</td>
</tr>
</tbody>
</table>

* Only strongly positive cells are shown.
* Significant difference from PBS-treated controls are shown in bold (n = 5; xx, p < 0.05; x, p < 0.02; **, p < 0.01; *, p < 0.001) (Student’s t-test).
FIGURE 5. Induction of Treg function by H471–94-tolerized pDC in vivo and autoantigen-specific expansion of Treg cells by DCs in vitro. A, Adoptive transfer of H471–94-tolerized pDC, but not non-pDC, increases suppressive ability of Treg cells in recipient mice in ex vivo suppression assay. Data as percent suppression (mean ± SEM) from three experiments (five mice per group). The purity of each subset of T cells was >90%.
B, For titration, Treg to target lupus Th were cocultured at 1:1, 1:2, 1:4; 1:10, and 1:40 ratios. The optimal results were seen at 1:4 ratio. C, In vitro, DCs expanded H471–94-specific CD4⁺CD25⁺foxp3⁺ Treg cells from whole T cells of low-dose H471–94-tolerized mice. DCs of both H4–tolerized and PBS-treated animals (H4 DC and PBS DC) expand foxp3⁺CD4⁺CD25⁺ T cells in the T cells (H4 T) of the H4–tolerized animal, in the presence of H4 (Ag), but not so in T cells from PBS-treated animals (PBS T). Numbers represent the mean percentage of three separate experiments (n = 5).
D, Results from the same three experiments were compared by histogram (CFSE dilution) for proliferated CD4⁺CD25⁺foxp3⁺ T cells. *, p < 0.001; **, p < 0.01; +, p < 0.05 (t test).
have class I epitope motifs, indicating that CD8+ Treg cells require additional factors to expand in vitro cultures.

Discussion

We found that low-dose tolerance with the dominant H471–94 peptide epitope could bring about a tolerogenic phenotype in a substantial fraction of pDC, which then induced Treg cells and suppressed Th17 inflammatory cells. The therapeutic benefit of H471–94 Peptide is further augmented by degeneracy of lupus autoimmune system for nucleosomal epitopes: a single peptide epitope from a histone in the nucleosome can be recognized by multiple autoimmune T cells of lupus with diverse receptors, and conversely, a single autoimmune T cell can recognize structurally different histone peptides (12, 14). Thus, a single peptide epitope can tolerate a spectrum of autoimmune Th cells and tolerizing one set of Th cells deprives help for multiple autoimmune B cells of lupus (tolerance spreading) (2, 3). Our studies indicate that s.c.-injected peptides spread systemically and were captured by splenic APC (DC). In another system, a tolerizing peptide injected s.c. spread through the body 16 times faster than an immunogenic peptide (36). We found that B cells are not involved here in induction of tolerance, which is in contrast to some studies (37) but consistent with others (38). Therefore, we focused on DC subsets.

Both pDC and non-pDC from H471–94-tolerized SNF1 mice on adoptive transfer reduced IgG autoantibodies significantly (p < 0.05–<0.001), but only pDC could suppress IFN-γ responses of lupus-prone recipient T cells to autoantigens, expand Treg cells with increased TGF-β production, and increase survival.

Our results indicate that H471–94 peptide in low-dose tolerance causes DCs, especially pDCs to produce increased amount of TGF-β, which is a critical factor for generation of Treg cells (39, 40). Although pDCs produce large amounts of type I IFNs in lupus (28), IFN-α mRNA levels were not changed by therapy. Also, we did not observe significant difference in IDO mRNA expression by low-dose tolerance, in contrast to other systems (41).

However, DCs from H471–94-tolerized mice upon stimulation by nucleosome or mitogens showed markedly suppressed production of IL-6. Ligation of TLRs on DCs stimulates production of IL-6, which overcomes CD4+CD25+ Treg cell-mediated suppression leading to T cell activation in vivo (24–26, 42). Our studies show that DC from low-dose H471–94-tolerized mice have an opposite phenotype with increased production of TGF-β and decreased IL-6 on stimulation with TLR ligands or the major autoantigen, nucleosomes; this tolerogenic phenotype was mediated by TLR9 binding of nucleosomal DNA. In accordance, TLR9 knockout lupus-prone MRL mice have a deficiency in Treg cells (43).

As recently described, exogenously added IL-6, with TGF-β, causes induction of Th17 cells that cause autoimmune tissue injury, and IL-6 inhibits the generation of Foxp3+ Treg cells induced by TGF-β (25, 26, 39, 40, 44). We show here that low-dose peptide tolerance of lupus could simultaneously induce Treg cells and suppress Th17 cells by increasing TGF-β and decreasing IL-6 production by DC. However, IL-23p19 levels were not changed in cultures of DCs from H471–94-tolerized mice. Th17 cells increased only in SNF1 lupus mice after stimulation with nucleosomes or H471–94, however, exogenously created polarizing cytokine conditions were not necessary. Moreover, PMA plus ionomycin stimulation alone could not bring out the splenic Th17 cells (data not shown). These results obtained with polyclonal peripheral T cells of nontransgenic, lupus-prone mice, in response to just one (albeit major) autoantigenic epitope, are notable because most Th17 cells migrate to target organs in autoimmune disease (Fig. 3F). The autoantigen-specific Th17 response was decreased up to 10-fold in low-dose peptide-tolerized mice (p < 0.001, Fig. 3, Table I). Low-dose H471–94 tolerance also prevented infiltration of Th17 cells in kidney (Fig. 3F), but we could observe some linear staining in glomeruli from both control and peptide-tolerized mice, probably due to expression of IL-17Rs on kidney cells (45).

Adoptive transfer of pDCs, but not non-pDCs from H471–94-injected mice increased suppressive activity of CD4+CD25+ Treg and CD8+ Treg cells in vivo. Moreover, the adaptive, Ag-specific Foxp3+ CD4+CD25+ Treg cells induced by the therapy could be expanded further ex vivo by cognate Ag (H471–94). Conversion of Ag-specific CD4+CD25+ T reg cells from CD45+CD25+ T cells and their expansion was described previously using TCR-transgenic cell with addition of a high amount of exogenous IL-2 and/or TGF-β (24, 40, 46, 47). Our studies provide direct evidence for induction of autoantigen, specifically induced Treg cells by low-dose peptide tolerance in complex setting of spontaneous autoimmune disease without the addition of high-dose exogenous IL-2 that may rescue premalignant T cells, or of TGF-β that in presence of high IL-6 in lupus could induce inflammatory Th17 cells (24–26).

Another group induced CD4+CD25+ Treg cells by continuous infusion of a model Ag in low doses using hemagglutinin-specific TCR-transgenic mouse system (48). However, continuous infusion of peptide indefinitely is not practical in humans. In lupus nephritis, striking therapeutic effect is achieved with biweekly s.c. injection of nucleosomal peptide epitopes in very low dose (2, 3, 15). In lupus patients, the most damaging side effects and morbidity occur from chronic life-long maintenance therapy with steroids and cytotoxic agents and, despite their use, flares and progression of renal disease occurs. Our therapy with H471–94 peptide has certain advantages: 1) it is an unaltered peptide ligand, being naturally occurring with evolutionarily conserved sequences, ubiquitous, expressed in the thymus during ontogeny (49), and not causing Th2 deviation (unlike altered peptide ligands) (50), and therefore, not associated with anaphylactic/allergic reactions; 2) it is effective at low doses and by s.c. administration in an animal model of lupus; 3) it generates long-lasting, Ag-specific regulatory T cells that suppress pathogenic autoantibody production and lupus nephritides; 4) it is cross-reactive, inducing “tolerance spreading” to other pathogenic T cell autoepitopes of lupus, but not to exogenous Ags; and 5) it is recognized by autoimmune T cells of all lupus patients tested irrespective of their HLA type.

Our peptide therapy might be most suitable for maintaining lupus patients after remission has been induced by more toxic or global immunosuppressive agents. Even apparently healthy subjects and family members of lupus patients, who might be at risk of developing lupus (as predicted by genetic and biomarkers), might benefit from the peptide therapy, because it repairs a defect in Treg cell deficiency in lupus (51–53). Importantly, these peptides appear to be effective even when the autoimmune disease is already established and restores normal lifespan in lupus mice (2, 3).

Disclosures

The authors have no financial conflict of interest.

References


