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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 Cells[1,2]

Hee-Kap Kang, Michael Liu, and Syamal K. Datta[3]

Subnanomolar doses of an unaltered, naturally occurring nucleosomal histone peptide epitope, $H4_{71–94}$, 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 $H4_{71–94}$ 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 $H4_{71–94}$ was effective even though the lupus immune system is spontaneously preprimed to react to the autoepitope. Thus, $H4_{71–94}$ 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 SNF_1 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, $H4_{71–94}$ is highly potent in low-dose tolerance therapy, because it cross-reactively suppresses autoimmunity to other pathogenic epitopes and whole nucleosomes (2, 3). Moreover, $H4_{71–94}$ also can suppress lupus via nasal tolerance (18). Importantly, $H4_{71–94}$ 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 SNF_1 and BWF_1 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 naive 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 $H4_{71–94}$ 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 $H4_{71–94}$ peptide induces $CD8^+$, and $CD4^+CD25^+$ adaptive regulatory T (Treg)

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[2] Preliminary results on the mechanism of low-dose peptide tolerance were presented as an abstract at the 2005 Annual Meeting of the American College of Rheumatology, Basic Research Conference, San Diego, CA, November 12–17 (H.-K. Kang, M. Liu, and S. K. Datta, 2005. Arthritis Rheum. 52:S32, Abstract BRC4).

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[4] Abbreviations used in this paper: MHC II, MHC class II; DC, dendritic cell; pDC, plasmacytoid DC; T reg, regulatory T cell; IDO, indoleamine 2,3 dioxygenase; MFI, mean fluorescence intensity.

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 SNF_1 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 SNF_1 females (nine mice per group) were injected s.c. three times with $H4_{71-94}$ peptide (1 μg/mouse) in PBS every 2 wk. The control group received only PBS. The mice were monitored weekly for proteinurea using Albustix (VWR Scientific).

Autoantibody quantitation

IgG class autoantibodies to ssDNA, dsDNA, histone, and nucleosome (histone-DNA complex) were measured by ELISA (7, 30). Subclasses of IgG autoantibodies were detected by ELISA using alkaline phosphatase-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates). One month after adoptive transfer of pDC or non-pDC, recipient SNF_1 mice (at 5.5 mo and 2 wk of age), with or without acceleration of disease as the case might be, were bled for autoantibody measurement.

Cell isolation

Total, $CD4^+$, and $CD8^+$ T cells from spleens were purified by using appropriate MACS isolation kits using magnetic bead-conjugated Abs specific to each Ag. $CD4^+CD25^+$ T cells were purified by a mouse regulatory T cell isolation kit according to the manufacturer (Miltenyi Biotec). For DC isolation, spleens were injected with DMEM containing 1 mg/ml collagenase (Worthington Biomedical) and 50 μg/ml DNase (Roche Applied Science) for digestion at 37°C for 45 min and then followed by washes in EDTA-containing buffer to prevent clumping as described (31). $CD11c^+$ DC, B cells, and macrophages were purified by using magnetic bead-conjugated Abs specific to CD11c, CD19, and CD11b, respectively. pDCs and non-pDCs were isolated by using the pDC isolation kit, according to the manufacturer (Miltenyi Biotec). Purity of all isolated cell subsets was >80–90%.

Ex vivo presentation assay to track s.c. injected peptide

We injected SNF_1 mice s.c. with a high dose (300 μg) of $H4_{71-94}$ peptide, following which we isolated DCs and B cells from their spleens at different time points between 6 and 48 h. The splenic DC or B cells were then cocultured with a highly sensitive T cell hybridoma, 102, which can detect $H4_{71-94}$ in attomole concentration (30). IL-2 production by T hybridomas was measured in culture supernatants. No exogenous peptide was added to cultures.

In vitro stimulation of purified DCs

We isolated DCs from low-dose $H4_{71-94}$-tolerized or PBS-treated mice and stimulated DC or DC subsets (3×10^5 cells/well of 96-well plates) with nucleosomes (10–50 μg/ml), LPS (5–1000 ng/ml) from Sigma-Aldrich, phosphorothioate CpG oligonucleotides 1585 (0.5–10 μg/ml) from Oligos Etc., or poly (I:C) (5–100 μg/ml) from InvivoGen, in 10% FBS containing DMEM for 48 or 90 h. For inhibition of TLRs, we stimulated purified DC or DC subsets (3×10^5cells/well) from $H4_{71-94}$-tolerized or PBS-treated SNF_1 mice with nucleosomes in the presence or absence of 0–30 μM TLR9 inhibitor (5′-TCCTGGAGGGGTTGT-3′), TLR7 inhibitor (5′-TGCTTGCAAGCTTGCAAGCA-3′) from Oligos Etc., or chloroquine from InvivoGen, for 48 or 90 h (32). We analyzed amounts of IFN-α, IL-6, IL-12p70, IL-17, IL-23p19, and TGF-β in culture supernatants by ELISA.

Adoptive transfer

To determine their ability to inhibit accelerated lupus nephritis, DC, DC subsets (pDC and non-PDC), or B cells were isolated by MACS from 3-mo-old donor SNF_1 mice 24 h after s.c. injection with $H4_{71-94}$ (300 μg/mouse) or PBS, and then immediately injected (1×10^6 cells I.V.) into 3-mo-old SNF_1, three times, at 2-wk intervals. One week later, the recipient mice were immunized with a lupus-accelerating nucleosomal peptide, $H1'_{22-42}$, in CFA. The animals were monitored for nephritis, survival, and IgG autoantibody levels 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 SNF_1 mice (five per group) were treated as above with or without immunization with $H1'_{22-42}$ in CFA. Ten days after the third transfer (1×10^6 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 tolerized 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 (1×10^6) from treated mice were cultured with irradiated (3000 rad) splenic APC (B cells, macrophages, and DC) from 1-mo-old SNF_1 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 immunospot scanning and analysis (Cellular Technology).

Suppression assay

Ten days after final (third) adoptive transfer of $H4_{71-94}$-tolerized or PBS-treated pDC or non-pDC, $CD4^+CD25^+$ and $CD8^+$ Treg cells (1×10^6) were isolated from recipient mice and the ability of Treg cells to directly inhibit IFN-γ responses of unmanipulated SNF_1 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 (1×10^6), T cell-depleted APC (5×10^5) plus T cells (1×10^6) from DC-recipient or unmanipulated SNF_1 mice were stimulated with $H4_{71-94}$ 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 and 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 (eBioscience).

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 (31). 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-MHC II (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 $H4_{71-94}$-tolerized or PBS-treated SNF_1 mice were cultured for 48 h with $H4_{71-94}$ or nucleosomes and then Golgi Stop (eBioscience) was added to cultures 12 h before cell staining. We also stimulated splenocytes from $H4_{71-94}$-tolerized or PBS-treated SNF_1 mice with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 nM; 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 $H4_{71-94}$-tolerized or PBS-treated SNF_1 mice were labeled with CFSE using the Vybrant Cell Tracer kit from Molecular Probes following

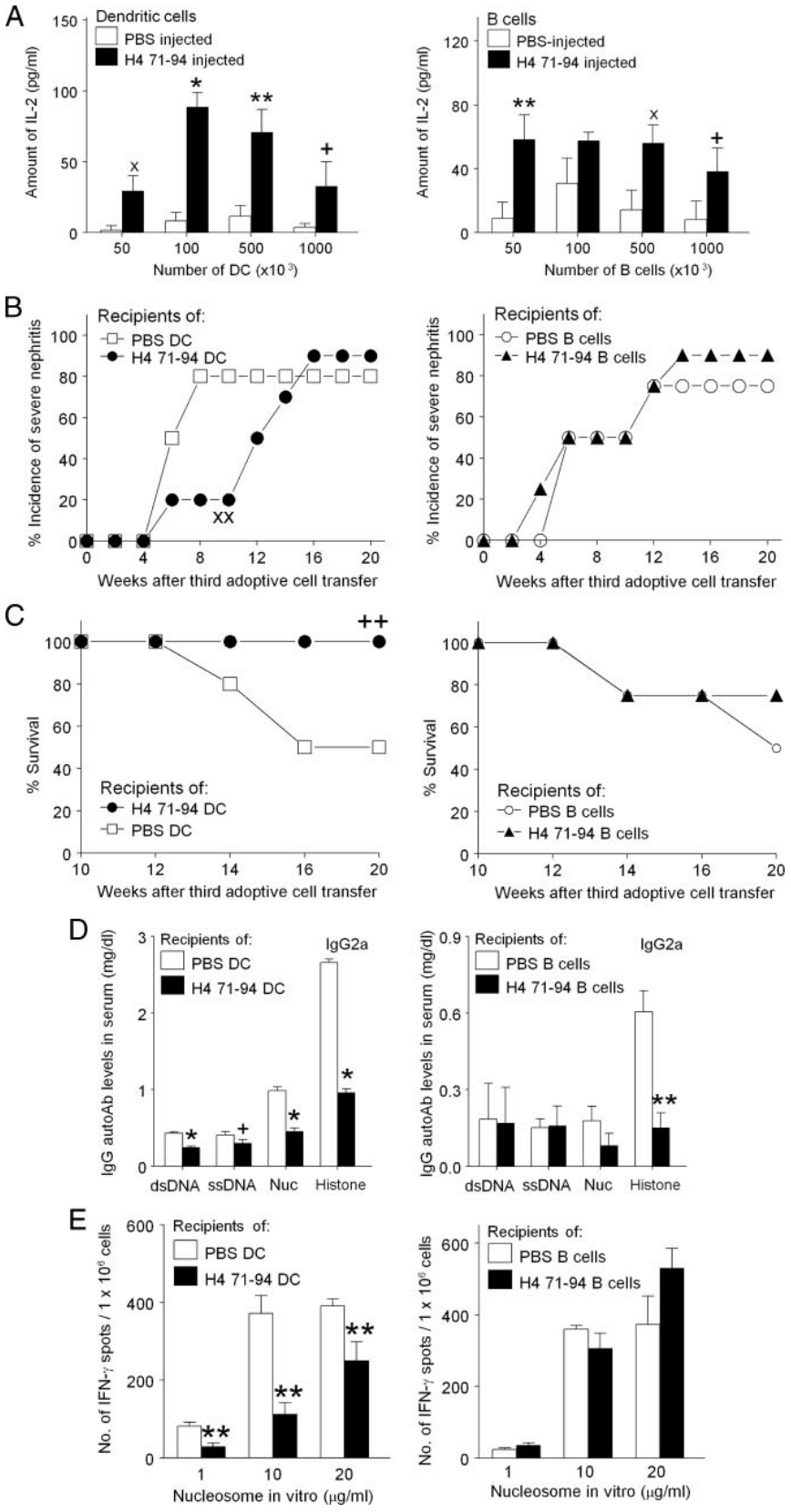

FIGURE 1. DCs are involved in peptide tolerance. *A*, Splenic DCs (*left*) take up s.c.-injected $H4_{71–94}$ peptide and present epitope to cognate, $H4_{71–94}$-specific T cell hybridoma more efficiently than B cells (*right*) in the ex vivo presentation assay (see *Materials and Methods*). Peak stimulatory ability seen at 24 h post-s.c. injection is shown. *B*, Adoptive transfer of the DC, but not B cells, of the $H4_{71–94}$-tolerized animal (H4 71-94 DC) delayed onset of severe nephritis in recipient SNF_1 mice with accelerated lupus. *C*, Adoptive transfer of DCs of the $H4_{71–94}$-tolerized animal, but not B cells, also prolonged the lifespan of the recipient animals as compared with recipients of the DCs or B cells of the control-treated animal. *D*, Adoptive transfer of $H4_{71–94}$-tolerized DC markedly decreased the pathogenic IgG2a subclass of anti-nuclear autoantibodies, but B cells only decreased IgG2a anti-histone autoantibody. SNF_1 mice were bled after 1 mo of adoptive transfer for autoantibody levels (mean ± SEM, milligrams per deciliter, $n = 5$). *E*, Adoptive transfer of the DCs, but not B cells, of the $H4_{71–94}$-tolerized animal decreased IFN-γ responses by lupus T cells in ELISPOT. Splenic T cells from SNF_1 mice that received DCs or B cells of the PBS-treated or $H4_{71–94}$-tolerized animal were challenged with nucleosomes in vitro. The baseline of IFN-γ responses was 6 ± 3 spots/1 × 10^6 T cells. Data from three experiments ($n = 5$). *B* and *C*, Log-rank test; xx, $p = 0.0124$; ++, $p = 0.0121$. All others: *t* test: ∗, $p < 0.001$ ∗∗, $p < 0.01$; x, $p < 0.02$; +, $p < 0.05$.

the manufacturer's protocol. CFSE-labeled T cells (1 × 10^6) were cocultured with $H4_{71–94}$-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 $CD4^+CD25^+foxp3^+$ cells or on $CD8^+TGF\text{-}\beta^+$ 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 $H4_{71-94}$ (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 $H4_{71-94}$-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 $\pm$ 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 $H4_{71-94}$, both splenic DCs and B cells stimulated a highly sensitive, $H4_{71-94}$-specific T cell hybridoma without further addition of the exogenous peptide (Fig. 1*A*). 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 $H4_{71-94}$-tolerized animals blocked lupus acceleration

Adoptive transfer of the splenic DCs of $H4_{71-94}$-tolerized animals delayed the onset of accelerated lupus nephritis in $H1'_{22-42}$-immunized SNF_1 mice (Fig. 1*B*) and prolonged survival (Fig. 1*C*). In the nephritis acceleration assay, young SNF_1 mice immunized with another nucleosomal epitope, $H1'_{22-42}$ in adjuvant 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 $H4_{71-94}$-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 $H4_{71-94}$-tolerized DCs, 100% of recipient mice had survived, whereas 50% of mice receiving control DCs were dead ($p < 0.01$). Transfer of $H4_{71-94}$-tolerized animal B cells had no significant effect (Fig. 1, *B* and *C*).

The DC and subsets from $H4_{71-94}$-tolerized animals diminished IgG autoantibodies and T cell responses to nucleosomes

Adoptive transfer of $H4_{71-94}$-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. 1*D*), but transfer of the B cells of the $H4_{71-94}$-tolerized animals did not, except for histones. Adoptive transfer of the DCs of $H4_{71-94}$-tolerized animals diminished recipient T cell responses to nucleosomes up to 70%, as compared with control recipients (Fig. 1*E*, $p < 0.01$), whereas transfer of $H4_{71-94}$-tolerized B cells did not (Fig. 1*E*, $p > 0.05$).

We further isolated subsets from $H4_{71-94}$ 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

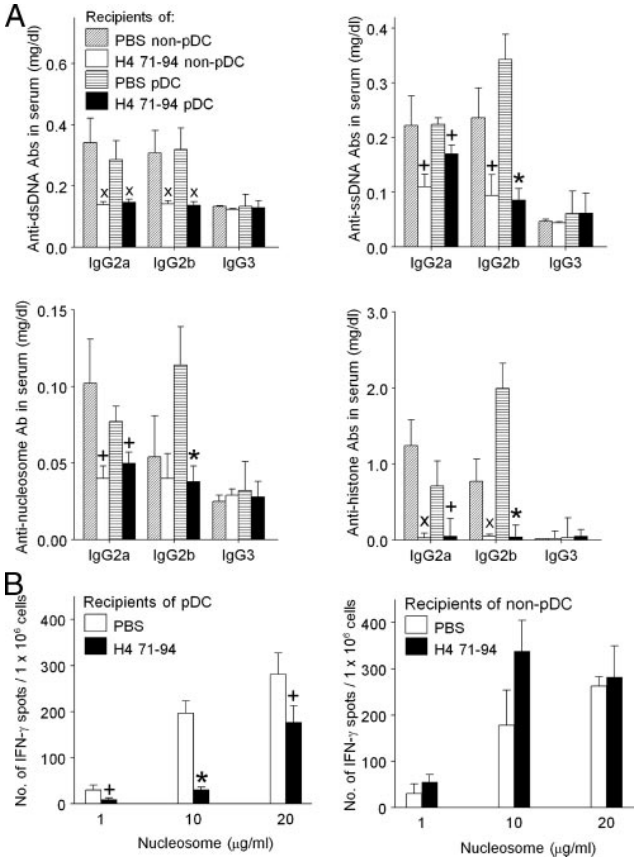

FIGURE 2. pDCs of the tolerized animal suppress pathogenic IgG subclasses of autoantibodies and autoimmune T cell responses. *A*, Adoptive transfer of $H4_{71-94}$-tolerized pDC or non-pDC markedly reduced the levels of pathogenic IgG subclasses of autoantibodies in recipient mice, as compared with controls. Percent reduction in autoantibody to dsDNA, ssDNA, nucleosomes, and histone, respectively, were as follows: IgG2a autoantibodies by $H4_{71-94}$-tolerized pDC: 49, 34, 36, and 93%; IgG2b by $H4_{71-94}$-tolerized pDC: 57, 75, 68, and 98%; IgG2a by $H4_{71-94}$-tolerized non-pDC: 60, 51, 61, and 98%; IgG2b by $H4_{71-94}$-tolerized non-pDC: 55, 65, 26, and 94% ($n = 5$). *B*, Adoptive transfer of $H4_{71-94}$-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 SNF_1 recipients of $H4_{71-94}$-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).

of $H4_{71-94}$ tolerized DCs reduced pathogenic IgG2a and IgG2b subclasses of autoantibodies in recipients (Fig. 2*A*, $p < 0.02$). Adoptive transfer of $H4_{71-94}$-tolerized pDC, but not non-pDC, markedly suppressed (up to 85%) the IFN-γ response of T cells of recipient mice on challenge with nucleosomes in vitro as compared with transfer of the pDC of the PBS-treated animals (Fig. 2*B*, $p < 0.001-0.05$). Thus, during the 5-mo observation, only three transfers of DCs from $H4_{71-94}$-tolerized mice could delay the incidence of severe nephritis, suppress responses of lupus T cells to nucleosomes, and reduce pathogenic IgG2a autoantibodies.

Transfer of the pDCs of $H4_{71-94}$-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 $H4_{71-94}$-tolerized pDCs

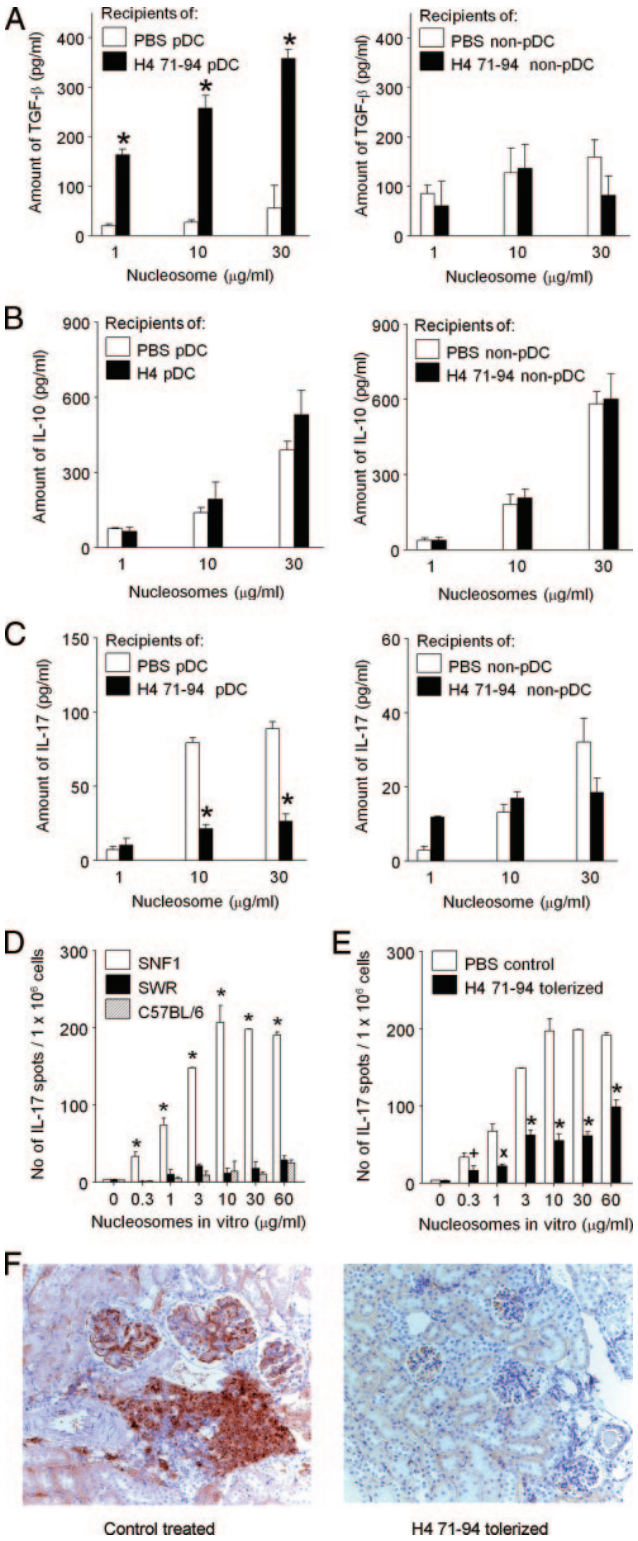

FIGURE 3. Adoptive transfer of the pDC of the $H4_{71-94}$-tolerized animal induced increased production of TGF-β by T cells of recipient mice in response to autoantigen. Whole splenocytes or T cell-depleted splenocytes from recipient SNF_1 mice of $H4_{71-94}$-tolerized or PBS-treated DC subsets were cultured in the presence of various concentrations of nucleosomes in vitro for 72–90 h and then the amount of cytokines in culture supernatants was measured by ELISA. *A*, Splenocytes from recipients of the $H4_{71-94}$-tolerized pDC produced markedly increased TGF-β, but T cell-depleted splenocytes from the same recipient mice did not (data not shown). Adoptive transfer of non-pDC showed no enhancing effect on TGF-β production. *B*, Adoptive transfer of $H4_{71-94}$-tolerized DC subsets did not increase IL-10 production by whole splenocytes or by T cell-depleted splenocytes from recipient mice (data not shown). *C*, Splenocytes from recipient mice of $H4_{71-94}$-tolerized pDC produced markedly decreased IL-17 in response to autoantigen, but transfer of non-pDC showed no significant effect. *D*, Unmanipulated, lupus-prone SNF_1 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 $H4_{71-94}$-tolerized SNF_1 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). *, $p < 0.001$; x, $p < 0.02$; +, $p < 0.05$ (*t* test).

Table I. *Intracellular staining of IL-17 on $CD4^+$-gated cells in spleen of 4.5-mo-old SNF_1 mice*

Ag In Vitro	PBS Treated (% positive)	H4 71-94 Tolerized (% positive)
PBS	2.66 ± 0.29	**0.36[a] ± 0.33**
$H4_{71-94}$	4.05 ± 0.47	**1.00 ± 0.45**
Nucleosomes	8.81 ± 0.2	**0.83 ± 0.53**

[a] Significant difference from controls are shown in bold ($n = 5$, all $p < 0.001$ (*t* test)).

(but not non-pDCs), in response to nucleosomes, as compared with that of PBS-treated pDCs (Fig. 3*A*). There were no significant differences among the recipient groups of DC subsets for IL-10 (Fig. 3*B*) or IL-4 production (data not shown). In contrast, IL-17 levels in nucleosome-stimulated cultures of splenocytes from SNF_1 recipients of the pDC (but not non-pDC) of $H4_{71-94}$-tolerized animals were markedly reduced, as compared with recipients of PBS-treated animal pDC (Fig. 3*C*, $p < 0.001$).

Unmanipulated lupus-prone SNF_1 mice had markedly increased Th17 response to nucleosome autoantigens, as compared with non-autoimmune, SWR, or C57BL/6 mice (Fig. 3*D*), and low-dose tolerance with $H4_{71-94}$ (1 μg) markedly reduced their IL-17 production in response to nucleosomes by ELISPOT (Fig. 3*E*). Flow cytometry also showed significantly reduced Th17 cells upon autoantigen stimulation in tolerized SNF_1 mice (Table I). We could not detect any significant Th17 cells in SNF_1 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 $H4_{71-94}$-tolerized mice, indicating that low-dose tolerance inhibited expansion and migration of activated Th17 cells to target organs (Fig. 3*F*).

Low-dose tolerance with $H4_{71-94}$ 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 $H4_{71-94}$-tolerized mice, as compared with controls (Fig. 4*A*), but no significant change in IDO mRNA ($p > 0.05$). In DC subsets, TGF-β mRNA in $H4_{71-94}$-tolerized pDCs was 4-fold higher than PBS control pDCs (Fig. 4*A*, $p < 0.001$), but no significant differences were seen in non-pDCs (Fig. 4*A*).

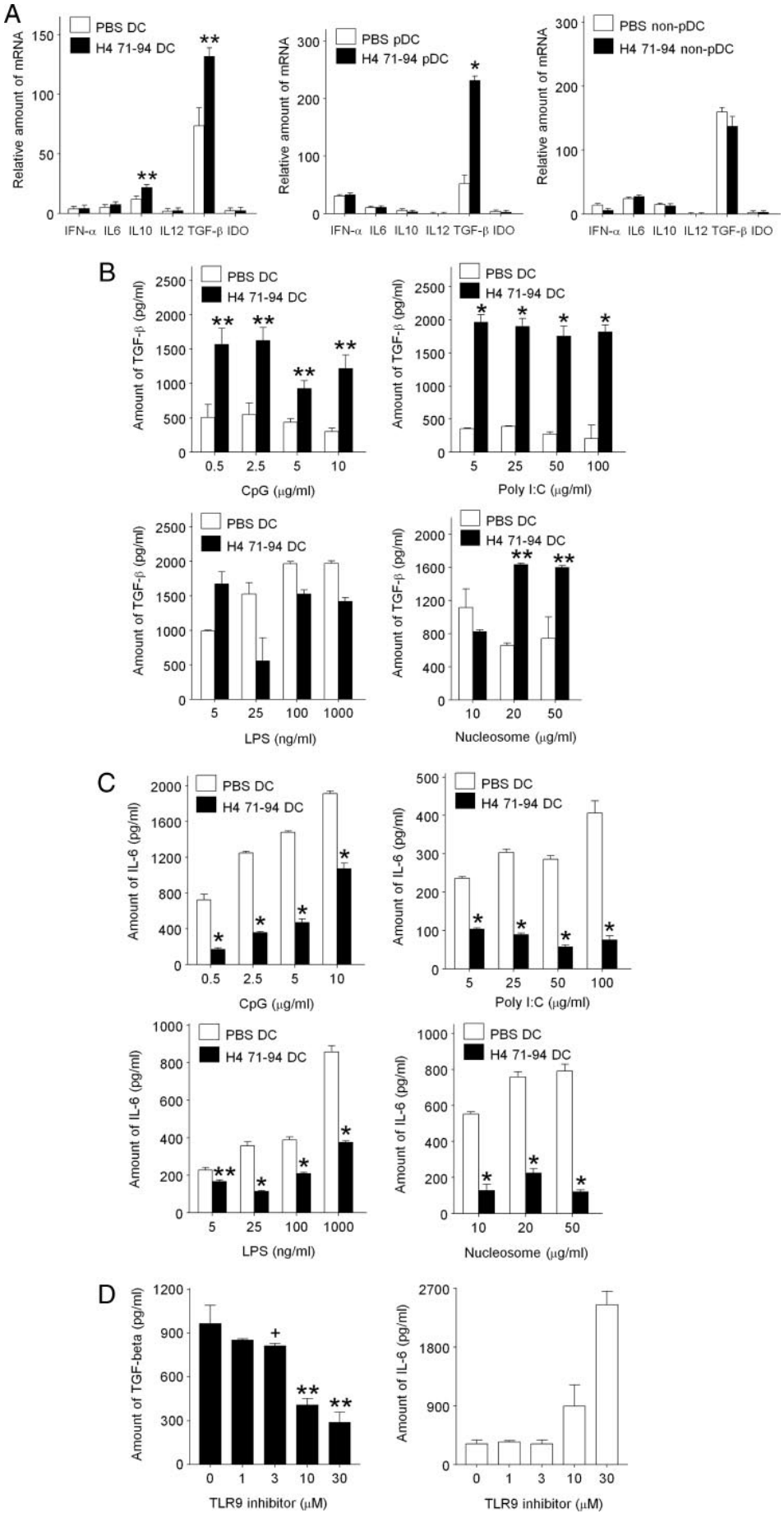

FIGURE 4. Low-dose $H4_{71–94}$ tolerance increases TGF-β mRNA expression in DCs, especially pDC. *A*, TGF-β mRNA (real-time PCR) was markedly increased in the pDC of the $H4_{71–94}$-tolerized animal (H4 71-94 pDC) as compared with the pDC of the PBS-treated animal (PBS pDC). Low-dose peptide tolerance 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 $H4_{71–94}$-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 $H4_{71–94}$-tolerized mice produced markedly decreased amount of IL-6 measured by ELISA. *D*, *Left*, Increased production of TGF-β by $H4_{71–94}$-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 $H4_{71–94}$-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).

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

Surface Markers	Fluorescence Intensity					
	pDC of PBS-treated animals			pDC of H4 71-94-tolerized animals		
	% Positive[a]	Mean	Median	% Positive	Mean	Median
CD86	14.24 ± 6	163.18 ± 2	43.56 ± 19	16.02 ± 8	**111.45[b] ± 16****	45.36 ± 19
CD40	43.82 ± 9	155.6 ± 10	107.46 ± 11	**24.39 ± 7[xx]**	**135.48 ± 4[xx]**	**61.25 ± 8****
CD80	14.74 ± 2	235.44 ± 25	36.78 ± 7	**10.84 ± 1[xx]**	**189.00 ± 8[xx]**	41.13 ± 7
MHC II	50.64 ± 1	789.75 ± 51	443.61 ± 115	51.51 ± 11	729.78 ± 236	510.33 ± 30
CD205	10.93 ± 6	127.31 ± 38	29.63 ± 15	12.85 ± 8	100.64 ± 53	29.53 ± 14
CD83	9.46 ± 6	430.98 ± 33	40.06 ± 15	7.75 ± 2	**172.78 ± 24***	35.73 ± 14
PD-L1	60.09 ± 14	292.15 ± 45	69.78 ± 4	60.20 ± 13	302.60 ± 47	**82.79 ± 3[x]**

[a] Only strongly positive cells are shown.
[b] 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)).

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. 2*B*, 3*A*, 4*A*, and 5*A* show that the tolerogenic effects were mainly due to pDC.

The low-dose tolerance regimen with $H4_{71-94}$ also increased production of TGF-β by DCs on stimulation in vitro with poly (I:C), CpG, or nucleosomes, but not LPS (Fig. 4*B*, $p < 0.001$–<0.01). In contrast, low-dose $H4_{71-94}$ tolerance markedly reduced IL-6 production by DCs stimulated by same agents (Fig. 4*C*, $p < 0.001$), as compared with control treatment. Amounts of IFN-α, IL-12, and IL-23 were not significantly different in DC cultures of $H4_{71-94}$-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 $H4_{71-94}$-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 $H4_{71-94}$-tolerized DCs on stimulation with nucleosomes in vitro (Fig. 4*D*, $p < 0.01$–0.05), but TLR7 inhibitor did not ($p > 0.05$, data not shown).

In contrast, the TLR9 inhibitor increased IL-6 production by $H4_{71-94}$-tolerized DCs on stimulation with nucleosomes in vitro (Fig. 4*D*). 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 ($0.3–1 \times 10^6$ cells/well).

pDCs of $H4_{71-94}$-tolerized animals have a tolerogenic phenotype

As shown in Table II, among the pDC of $H4_{71-94}$-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 $H4_{71-94}$-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 $H4_{71-94}$-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 $H4_{71-94}$-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 $H4_{71-94}$-tolerized animals had increased TGF-β^+ cells ($45.20 \pm 3\%$, $p < 0.01$), 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 $H4_{71-94}$-tolerized mice ($p < 0.01$). Furthermore, mean and median fluorescence of TGF-β^+ pDC by low-dose $H4_{71-94}$ tolerance were 2.5- and 1.96-fold higher than those of non-pDC (all $p < 0.05$).

Transfer of $H4_{71-94}$-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 $H4_{71-94}$-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 SNF_1 mice, in the presence of nucleosomes. As compared with CD4$^+$CD25$^+$ and CD8$^+$ T cells from recipients of PBS-treated animal pDC (Fig. 5*A*, $p < 0.001$), both of those cell subsets from recipients of $H4_{71-94}$-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. 5*B*). However, adoptive transfer of $H4_{71-94}$-tolerized non-pDC did not increase suppressive function (Fig. 5*A*, $p > 0.05$).

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 $H4_{71-94}$-tolerized or PBS-treated SNF_1 mice were cocultured for 72 h with DCs from low-dose $H4_{71-94}$-tolerized or PBS-treated SNF_1 mice in the presence of $H4_{71-94}$ 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 $H4_{71-94}$-tolerized mice proliferated upon coculture with $H4_{71-94}$-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 $H4_{71-94}$-tolerized DC and PBS-treated DC expanded relatively more CD4$^+$CD25$^+$foxp3$^+$Treg cells from low-dose $H4_{71-94}$-tolerized mice with addition of $H4_{71-94}$ peptide than with nucleosomes.

Under similar conditions, CD8$^+$ Treg cells were not expanded by DC with nucleosomes or $H4_{71-94}$, although the autoantigens

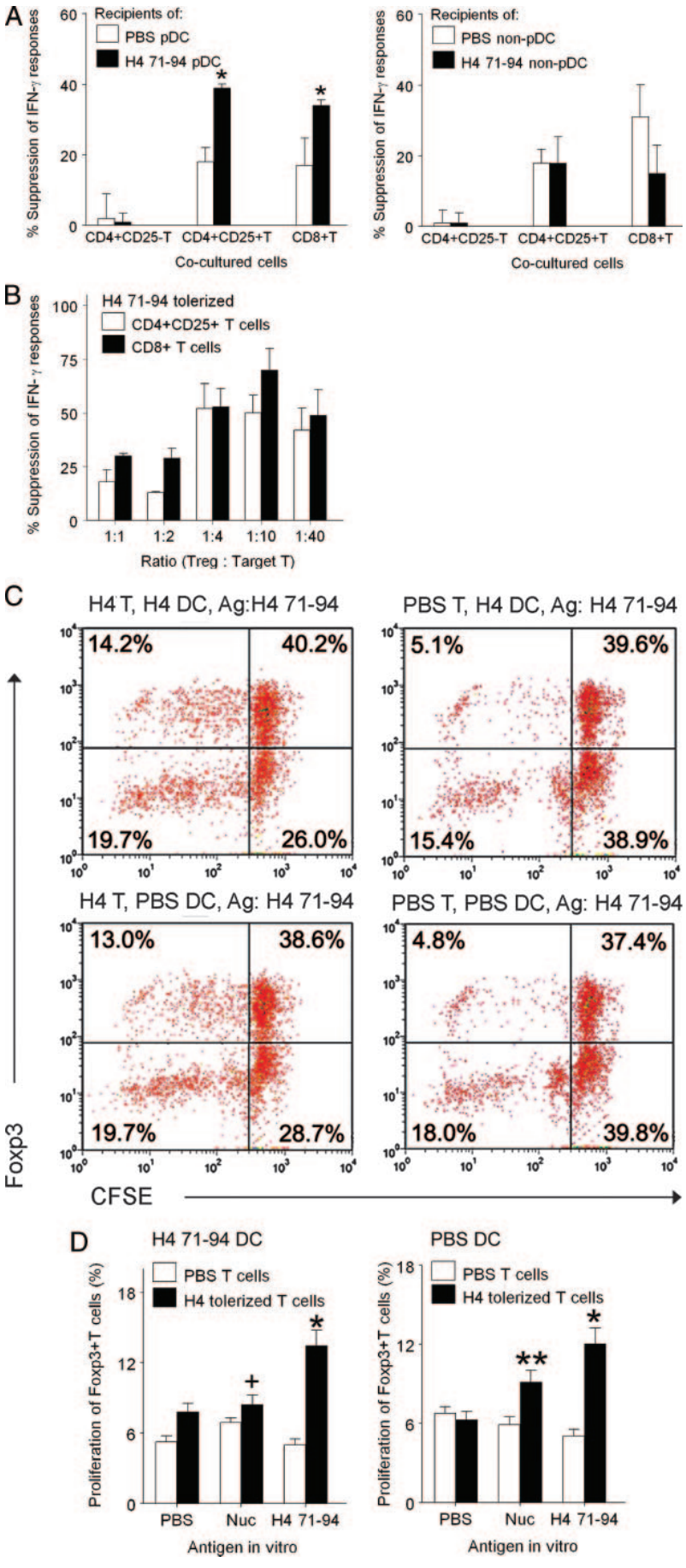

FIGURE 5. Induction of Treg function by $H4_{71–94}$-tolerized pDC in vivo and autoantigen-specific expansion of Treg cells by DCs in vitro. *A*, Adoptive transfer of $H4_{71–94}$-tolerized pDC, but not non-pDC, increases suppressive ability of Treg cells in recipient mice in ex vivo suppression assay. 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 $H4_{71–94}$-specific $CD4^+CD25^+foxp3^+$Treg cells from whole T cells of low-dose $H4_{71–94}$-tolerized mice. DCs of both $H4_{71–94}$-tolerized and PBS-treated animals (H4 DC and PBS DC) expand $foxp3^+CD4^+CD25^+$ T cells in the T cells (H4 T) of the $H4_{71–94}$-tolerized animal, in the presence of $H4_{71–94}$ (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 $H4_{71–94}$ 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 $H4_{71–94}$ 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 tolerize 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 $H4_{71–94}$-tolerized SNF_1 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 $H4_{71–94}$ 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 $H4_{71–94}$-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 $H4_{71–94}$-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 $H4_{71–94}$-tolerized mice. Th17 cells increased only in SNF_1 lupus mice after stimulation with nucleosomes or $H4_{71–94}$, 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 Th 17 cells migrate to target organs in autoimmune disease (Fig. 3*F*). The autoantigen-specific Th 17 response was decreased up to 10-fold in low-dose peptide-tolerized mice ($p < 0.001$, Fig. 3, Table I). Low-dose $H4_{71–94}$ tolerance also prevented infiltration of Th17 cells in kidney (Fig. 3*F*), 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 $H4_{71–94}$-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 ($H4_{71–94}$). Conversion of Ag-specific $CD4^+CD25^+$ Treg cells from $CD45^+CD25^-$ T cells and their expansion was described previously using TCR-transgenic cells 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 $H4_{71–94}$ 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 nephritis; 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.

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