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Histone-Specific CD4$^+$ T Cell Plasticity in Active and Quiescent Systemic Lupus Erythematosus

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Objective. The aim of this study was to assess whether circulating histone-specific T cells represent tools for precision medicine in systemic lupus erythematosus (SLE).

Methods. Seroprevalence of autoantibodies and HLA-DR beta (DRB) 1 profile were assessed among 185 patients with SLE and combined with bioinformatics and literature evidence to identify HLA–peptide autoepitope couples for ex vivo detection of antigen-specific T cells through flow cytometry. T cell differentiation and polarization was investigated in patients with SLE, patients with Takayasu arteritis, and healthy controls carrying HLA-DRB1*03:01 and/or HLA-DRB1*11:01. SLE Disease Activity Index 2000 and Lupus Low Disease Activity State were used to estimate disease activity and remission.

Results. Histone-specific CD4$^+$ T cells were selectively detected in patients with SLE. Among patients with a history of anti-DNA antibodies, 77% had detectable histone-specific T cells, whereas 50% had lymphocytes releasing cytokines or upregulating activation markers after in vitro challenge with histone peptide antigens. Histone-specific regulatory and effector T helper (Th) 1-, Th2-, and atypical Th1/Th17 (Th1*)-polarized cells were significantly more abundant in patients with SLE with quiescent disease. In contrast, total Th1-, Th2-, and Th1*-polarized and regulatory T cells were similarly represented between patients and controls or patients with SLE with active versus quiescent disease. Histone-specific effector memory T cells accumulated in the blood of patients with quiescent SLE, whereas total effector memory T cell counts did not change. Immunosuppressants were associated with expanded CD4$^+$ histone-specific naive T (T_N) and terminally differentiated T cells.

Conclusion. Histone-specific T cells are selectively detected in patients with SLE, and their concentration in the blood varies with disease activity, suggesting that they represent innovative tools for patient stratification and therapy.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease characterized by clinical variability. Genetically determined or acquired defects of the innate and adaptive immune response concur to the expression of SLE at a clinical level and possibly account for failure of most clinical/diagnostic tools and treatments to prevent chronic disability.[1,2] B cell and antibody responses have extensively been investigated in patients with SLE. Autoantibodies targeting nuclear components, including DNA and chromatin constituents, prompt organ damage by promoting complement activation and monocyte recruitment at sites of inflammation. They result from isotype switching and affinity maturation, events that depend on B cell interaction with antigen-specific T cells, and strengthen upstream innate and adaptive immune mechanisms by opsonizing nuclear components and inducing antiviral-like interferon (IFN) α-driven anti–nucleic acid responses.[3] Autoantibody responses against nuclear

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constituents represent the backbone of current diagnostic tools,[1,4] and antinuclear autoantibodies are an obligatory entry criterion for the 2019 EULAR/American College of Rheumatology (ACR) classification criteria for patients with SLE.[5] The B cell/IFNα axis represents the main target of current molecularly targeted treatments for patients with SLE. Yet, only a fraction of patients with SLE might be adequately monitored and treated based on tracking and modulation of their autoantibody profile.[6]

T cell responses have less been investigated in patients with SLE despite their apical role in shaping antigen-specific immune responses. The CD3/T cell receptor (TCR) signaling complex undergoes genetically or epigenetically determined alterations in patients with SLE,[7,8] and activity- and phenotype-related numerical variations in lymphocyte counts and in the relative distribution of $CD4^+$ and $CD8^+$ T cell with distinct differentiation (stemness) and polarization features have been observed.[9–16] Cytokine release and activation marker expression occur after in vitro stimulation of T cells from patients with SLE with chromatin components,[17–19] suggesting the existence of circulating antigen-specific T cells, whose relative abundance and functional characteristics cannot, however, be finely dissected by this approach. Here, we verified whether the ex vivo visualization of major histocompatibility complex (MHC)-restricted histone antigen-specific $CD4^+$ T cells by multiparameter flow cytometry in patients with SLE could be used to implement molecular profiling for precision medicine in patients with SLE.

PATIENTS AND METHODS

Patients and controls. This research was part of the "Autoimmuno-Mol" research protocol, approved by the IRCCS Ospedale San Raffaele Institutional Review Board (reference number 02/2013/INT) and conforming to the Declaration of Helsinki. Upon informed consent under the "Autoimmuno-Mol" research protocol, 185 consecutive patients with SLE who were classified according to the 2012 Systemic Lupus Erythematosus International Collaborating Clinics criteria[20,21] were enrolled and genotyped for their HLA-DR beta (DRB) 1 profile (Supplementary Methods and Supplementary Figure 1). Blood samples were used to obtain DNA, peripheral blood mononuclear cells (PBMCs) and sera. A total of 86 healthy controls (HCs) and 80 patients with Takayasu arteritis (TAK), classified according to the 1996 criteria from Sharma et al,[22] served as controls. TAK was chosen, being comparable to SLE in terms of demographics and pathogenic role of T cells but unlikely to overlap clinically with SLE.

Disease history and trends of laboratory features over time of patients with SLE and patients with TAK were retrospectively retrieved from clinical charts. TAK activity was measured through the Indian Takayasu Clinical Activity Score 2010.[23] Regarding patients with SLE, clinical, laboratory, and treatment features at time of enrollment were collected through a dedicated in-house software as previously described.[24] Disease activity was measured by using the SLE Disease Activity Index 2000 (SLEDAI-2K).[25] The Lupus Low Disease Activity State (LLDAS) was used as a surrogate of remission.[26] Patients' perceptions on the impact of SLE on their health were captured through a 0 to 10 numerical rating scale. Chronic damage was quantitated through the Systemic Lupus Erythematosus International Collaborating Clinics/ACR Damage Index.[27] Laboratory test data included complete blood cell counts, liver and renal function tests, urine analysis, erythrocyte sedimentation rate and C-reactive protein levels, complement C3 and C4 levels, and anti-DNA antibody (ADNA) titers at time of enrollment. The presence of ADNAs was assessed by semiquantitative enzyme-linked immunosorbent assay (ELISA; Cusabio). Anti-histone antibodies were also quantitated through ELISA (Bluegene), and a cutoff of normality was set above the 95th percentile of HC values.

MHC–peptide multimers. We used MHC–peptide multimer staining to detect antigen-specific T cells in blood. To identify suitable MHC–peptide combinations, a stepwise algorithm was applied integrating clinical information, bioinformatics, and evidence from the literature. Based on cohort seroprevalence (Supplementary Table 1), we ruled out the Smith antigen, beta-2-glycoprotein I, and U1-RNP as candidate antigens and focused on chromatin components. Anti-DNA immunity was assumed to be effectively surrogated by anti-nucleosome reactivity.[18,19,28] Therefore, histone proteins were selected as candidate antigens. HLA restriction was limited to two HLA-DRB1 variants (HLA-DRB1*03:01 and HLA-DRB1*11:01) showing (a) high prevalence in the study cohort (Supplementary Table 2), (b) relatively high representation in the general population,[29] and (c) pathogenic relevance for SLE.[30,31] Finally, histone H3 and H4 peptide sequences were tested for potential epitopes able to effectively bind HLA-DRB1*03:01 and/or HLA-DRB1*11:01 by using the Immune Epitope Database and Analysis Resource (www.iedb.org).[32] Only peptides with percentile ranks below the 25th were considered. Histone H4–related GLIYEETRGVLKVFL (peptides 49–63) sequence was prioritized over other sequences as an HLA-DRB1*03:01 binder because of available evidence of T cell reactogenicity (including enhanced help to ADNAs releasing B cells) in patients with SLE from the literature.[19] VVYALKRQGRTLYGFG (histone H4 87–102) was also identified as an HLA-DRB1*03:01 binder, and LPFQRLMREIAQD (histone H3 66–78) was found to be a potential HLA-DRB1*11:01 binder. Fluorochrome-conjugated DRB1*03:01 and DRB1*11:01 tetramers bound to GLIYEETRGVLKVFL (histone H4 49–63)[19] and LPFQRLMREIAQD (histone H3 66–78) peptides, respectively, were purchased from ProImmune.

Ex vivo T cell visualization. PBMCs were resuspended in RPMI 1640 medium (Thermo Fisher Scientific, Gibco) containing 0.1% gentamycin and 10% fetal bovine serum (culture medium). After overnight incubation, cells were placed in polypropylene

tubes and stained with anti-CD3, -CD4, -CD8, -CD45RA, -CD62L, and -CD95 antibodies (panel A) ± MHC tetramers[9,33,34] and with anti-CD3, -CD4, -CD25, -CD127, -CD183 (=CXCR3), -CD194 (=CCR4), and -CD196 (=CCR6) antibodies (panel B) ± MHC tetramers (Supplementary Figures 2 and 3).[9,16,35] Vital staining was performed using Zombie Aqua (BioLegend). All cells were treated with 10 μM dasatinib 1 μL/50 μL cells for 15 minutes at 37°C before staining. Stained cells were analyzed with a Beckman Coulter Navios flow cytometer, and list mode data files were elaborated through FCS Express version 7. Additional details on cell staining protocols and gating strategies are provided in Supplementary Methods. A single HC with both HLA-DRB1*03:01 and HLA-DRB1*11:01 was tested with both MHC tetramers. All other patients were tested with the MHC tetramer corresponding to their HLA-DRB1 genotype. Total antigen-specific $CD4^+$ T cell frequencies were calculated by subtracting the background $CD4^-$ MHC tetramer–positive events from the number of $CD4^+$ and MHC tetramer–positive events.[36]

T cell activation assays. Histone H4 49–63 (GLIYEETRGVLKVFL) and histone H3 66–78 (LPFQRLMREIAQD) synthetic sequences were purchased from Biomatik. PBMCs from a subset of 13 patients with SLE and 5 HCs were challenged with the relevant peptide antigen (20 μg/mL). Unstimulated cells served as negative controls and phytohemagglutinin-stimulated cells as positive controls. After 24 hours, supernatants were collected and eventually analyzed for the concentration of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IFNγ, TNFα, and IL-22 through a multiplex cytokine bead array (BioLegend LEGENDplex; Supplementary Methods). Lymphocytes were stained with a vital dye (Zombie Aqua, BioLegend), followed by anti-CD3, CD4, CD69, and OX40 fluorochrome-bound antibodies and analyzed by flow cytometry as described above. Patients whose $CD4^+$ cells expressed the activation markers CD69 and OX40 upon challenge[37,38] and/or with stimulated release of at least 110% of any cytokine with respect to the unstimulated control[39] were considered as responders. Quantitatively lower cytokine/activation markers responses after stimulation than under unstimulated conditions were defined as suppressed responses.[18]

Statistical analysis. Receiver operating characteristics (ROC) curves were built to identify diagnostic thresholds for detecting patients with a history of positive ADNAs. Normal distribution of continuous variables was assessed through the Shapiro–Wilk test. Nonnormally distributed quantitative variables were compared among two or more groups by using the Mann–Whitney or Kruskal–Wallis test, respectively. Variations in continuous variables among the same patients were analyzed through the Wilcoxon's matched-pairs signed-rank test. The frequencies of categorical values among groups were compared by using the chi-square test with Fisher's exact correction. Correlations among nonnormally distributed variables were assessed through the Spearman's test. Statistical analyses were performed with StataCorp Stata version 15. Data are expressed as median (interquartile range [IQR]) unless otherwise specified. The data underlying this article will be shared on reasonable request to the corresponding author.

RESULTS

Genotype frequencies and clinical features. Of 185 enrolled patients with SLE, 159 were women (86%) and 144 (78%) had positive ADNAs in their history (Supplementary Table 1). HLA-DRB1*03:01 (34%), HLA-DRB1*07:01 (22%), HLA-DRB1*11:01 (15%), HLA-DRB1*15:01 (15%), and HLA-DRB1*01:01 (14%) were the five most represented HLA-DRB1 alleles (Supplementary Table 2). HLA-DRB1*03:01 carriers were significantly more frequent among patients with SLE than among patients with TAK (11%; $\chi^2 = 14.114$; $P < 0.001$) and HCs (17%; $\chi^2 = 7.455$; $P = 0.006$), as expected,[31] whereas DRB1*11:01 was also highly represented in patients with TAK (19%) and HCs (15%). Among patients with DRB1*03:01 and/or DRB1*11:01 alleles (90 of 185 patients with SLE, 24 of 80 patients with TAK, and 28 of 86 HCs; Supplementary Figure 1), 32 patients with SLE, 11 patients with TAK, and 10 HCs (one of whom had both alleles) of comparable sex and age distribution (Table 1) had sufficient cell aliquots for T cell assays. Their individual demographics, disease activity, and treatment features at enrollment are reported in Supplementary Table 3. Globally, 23 patients with SLE (72%) and 7 patients with TAK (64%) were in remission. Five patients with SLE in remission were also tested in an active phase of the disease (Supplementary Figure 4). Their samples collected during active disease were excluded from the other T cell studies. The timeframe between the two samples ranged between 1 and 25 months. The median (IQR) SLEDAI-2K was 2 (2–4) in patients with SLE. The median (IQR) daily prednisone dose was 1 mg (0–4 mg) among patients with SLE and 5 mg (3–5 mg) among patients with TAK. A total of 18 patients with SLE (56%) and 10 patients with TAK (91%) were taking immunosuppressants (Supplementary Table 3). Twenty-six patients with SLE (81%) had a history of ADNAs. ADNAs were detected in 10 of 37 samples from patients with SLE (27%), 1 of 11 samples from patients with TAK (9%), and 0 of 10 samples from HCs at time of clinical assessment. Patients with anti-histone antibodies accounted for 6% of total patients with SLE and 22% of patients with active disease. Anti-histone antibody concentrations correlated with SLEDAI-2K (rho = 0.490; $P = 0.004$) and were higher in patients with a history of ADNAs (298 ng/mL [274–321 ng/mL] vs 252 ng/mL [237–259 ng/mL] in patients with negative ADNAs; $P = 0.038$) and in those not fulfilling LLDAS criteria (309 ng/mL [300–365 ng/mL] vs 282 ng/mL [251–299 ng/mL] in patients with LLDAS; $P = 0.020$). Additional clinical and serological features at the time of enrollment and in patients' history are reported in

Table 1. Patients tested for histone-specific CD4+ T cells*

Characteristics	Patients with SLE (n = 32)	Patients with TAK (n = 11)	HCs (n = 10)[a]
Female, n (%)	28 (88)	10 (91)	9 (90)
Age, median (IQR), y	41 (34–51)	44 (36–47)	41 (34–51)
HLA-DRB1*03:01, n (%)	15 (47)	4 (36)	6 (60)
HLA-DRB1*11:01, n (%)	19 (59)	7 (64)	5 (50)
Disease duration, median (IQR), y	17 (9–23)	10 (7–15)	NA
SLE disease activity			
SLEDAI-2K, median (IQR)	2 (2–4)	NA	NA
LLDAS, n (%)	23 (72)	NA	NA
TAK disease activity ITAS, median (IQR)	NA	0 (0–1)	NA
Clinical impression, n (%)			
Active	NA	1 (9)	NA
Smoldering	NA	3 (27)	NA
Remission	NA	7 (64)	NA
Immunomodulant and immunosuppressive treatments			
HCQ, n (%)	28 (88)	0 (0)	0 (0)
PDN, median (IQR), mg	0.6 (0–1.3)	5 (0–5)	0 (0–0)
AZA, n (%)	2 (6)	2 (18)	0 (0)
MMF, n (%)	12 (38)	0 (0)	0 (0)
MTX, n (%)	3 (9)	6 (55)	0 (0)
IFX, n (%)	0 (0)	4 (36)	0 (0)
TCZ, n (%)	0 (0)	1 (9)	0 (0)
ABA, n (%)	0 (0)	1 (9)	0 (0)
Others,[b] n (%)	1 (3)	1 (9)	0 (0)

* ABA, abatacept; AZA, azathioprine; DRB, DR beta; HC, healthy control; HCQ, hydroxychloroquine; IFX, infliximab; IQR, interquartile range; ITAS, Indian Takayasu Clinical Activity Score; LLDAS, Lupus Low Disease Activity State; MMF, mycophenolate mofetil; MTX, methotrexate; NA, not applicable; PDN, prednisone; SLE, systemic lupus erythematosus; SLEDAI-2K, SLE Disease Activity Index 2000; TAK, Takayasu arteritis; TCZ, tocilizumab.
[a] One of the 10 HCs had both HLA-DRB1*03:01 and HLA-DRB1*11:01 alleles and was therefore tested for antigen-specific T cells and T cell activation to both HLA-DRB1*03:01– and HLA-DRB1*11:01–restricted epitopes.
[b] One patient with SLE received intravenous immunoglobulins, and one patient with TAK received sirolimus.

Supplementary Table 4. No patient with TAK had positive anti-histone antibodies.

Histone-specific T cells. Histone-specific CD4+ T cells were significantly higher in patients with SLE than in HCs and patients with TAK by orders of magnitude (Figure 1A–E; Supplementary Table 5). In patients with SLE, a median of 0.06% (0.03–0.43%) CD4+ T cells were histone-specific T cells, with 14% of patients showing histone-specific CD4+ T cells exceeding 1% of total CD4+ T cells. There was no difference in the frequency and size of histone-specific CD4+ T cells detected with the use of HLA-DRB1*03:01 or HLA-DRB1*11:01 tetramers. There was also no difference in the total number of acquired cell events among groups (data not shown). To define a clinically meaningful threshold of significance for histone-specific CD4+ T cell counts, ROC curves were built to test the diagnostic ability of this biomarker against the gold-standard, positive ADNA history. Histone-specific CD4+ T cells levels accounted for 78.5% of the total variability underlying a history of positive ADNAs across patients and HCs ($P = 0.001$; Supplementary Figure 5). Consistently, histone-specific CD4+ T cells above the ROC curve–defined threshold (seven MHC tetramer–positive events/sample) were significantly more frequent in patients with a history of ADNAs (20 of 26, 77%, all with SLE) than in patients with negative ADNAs (6 of 27, 22%; $\chi^2 = 15.86$; $P < 0.001$). Exploratory analyses in a subset of patients with SLE and HCs showed an in vitro response of PBMCs to histones in up to 54% of patients with SLE and up to 56% of patients with a history of ADNAs. Notably, stimulation with histone peptides elicited suppressed responses in all HCs (Table 2; Supplementary Figures 6 and 7). A total of 15 of 20 patients with a history of ADNAs and positive histone-specific CD4+ T cells had negative ADNAs at the time of venipuncture. Three patients with no history of ADNAs but with positive histone-specific T cells had SLE. Evidence for in vitro T cell reactivity to histone peptides was found for all three of these patients. In general, 23 of 32 patients with SLE (72%), 3 of 11 patients with TAK (27%), and no HCs had histone-specific CD4+ T cells. The frequency and count of histone-specific CD4+ T cells did not differ by ADNA status at the time of sampling or by the presence versus absence of any clinical or serological SLE manifestation, including lymphopenia. There was no correlation among levels of total histone-specific CD4+ T cells, anti-histone antibodies, and ADNAs at the time of sampling.

In patients with SLE, histone-specific CD4+ T cells were detected across all memory subsets, with a similar proportion of naive and of memory (including stem cell memory, central memory, effector memory, and terminally differentiated effector memory) cells (Figure 1F). Furthermore, histone-specific CD4+

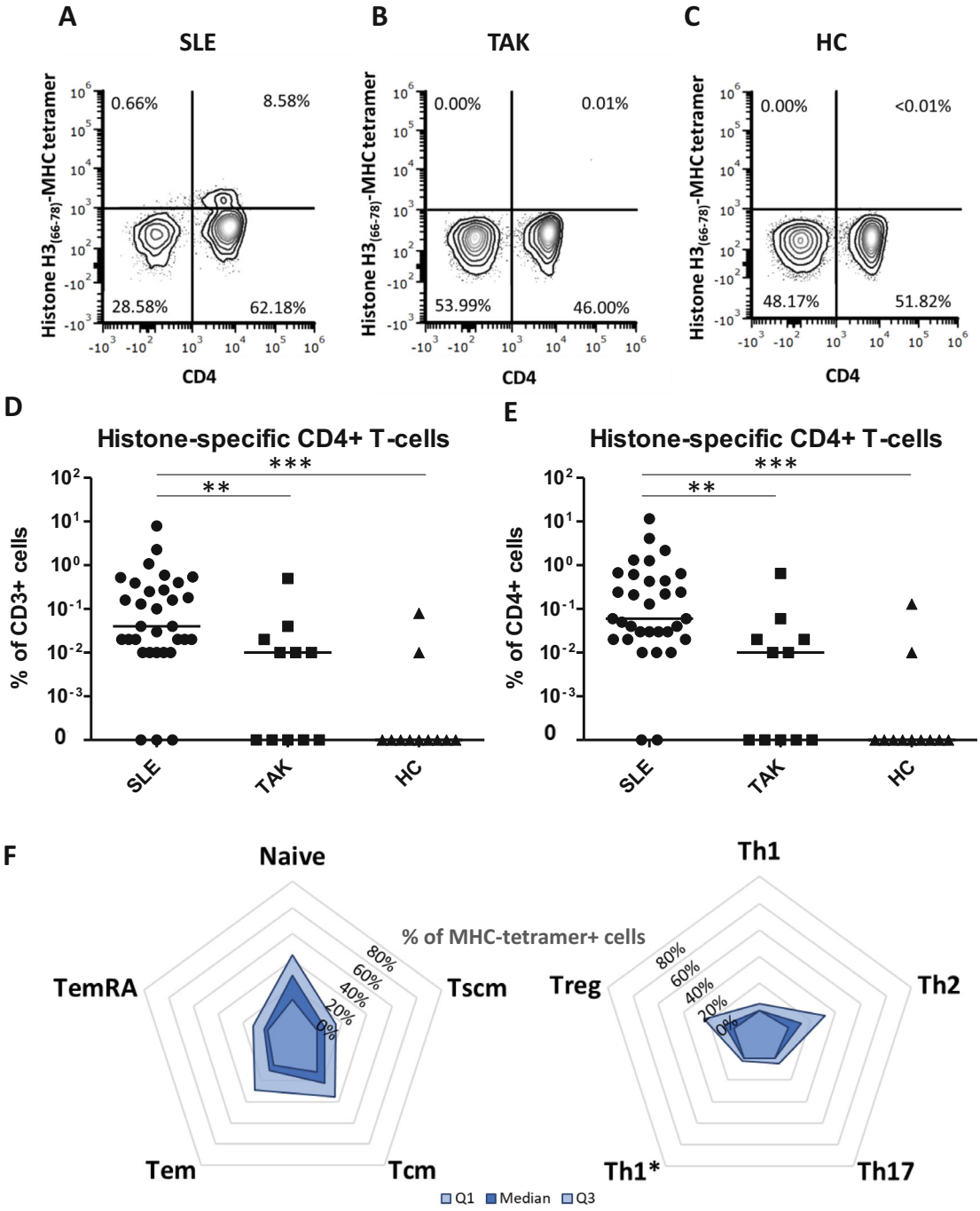

Figure 1. Histone-specific CD4$^+$ T cells in patients with SLE and HCs. (A–E) Histone-specific CD4$^+$ T cell frequency in patients and HCs and (F) their differentiation/polarization profile in patients with SLE are depicted. (A–C) Contour plots showing the magnitude of T cells stained by LPFQRLMREIAQD (histone $H3_{66–78}$) peptide-bound MHC tetramers (y axis) among the total CD4$^+$ T cells (x axis) in three representative (A) patients with SLE, (B) patients with TAK, and (C) individuals with no disease(all HLA-DRB1*11:01 allele carriers). (D and E) Dot plots showing the frequency of histone-specific CD4$^+$ T cells among (D) CD3$^+$ and (E) CD4$^+$ cells in a logarithmic scale. Histone-specific CD4$^+$ T cells were more frequent in patients with SLE than in patients with TAK and HCs. $^{**}P < 0.010$, $^{***}P < 0.001$. (F) Radial plots showing the relative frequencies of MHC tetramer–positive CD4$^+$ T cells with distinct differentiation and polarization profiles in patients with SLE. Inmost and outmost colored polygons represent Q1 and Q3 for each parameter, respectively, whereas middle polygons depict median values. Early stages of differentiation were slightly more expanded, whereas skewness toward Th2 and Treg was observed in terms of polarization. HC, healthy control; MHC, major histocompatibility complex; Q1, first quartile; Q3, third quartile; SLE, systemic lupus erythematosus; TAK, Takayasu arteritis; Tcm, central memory T; Tem, effector memory T; TemRA, terminally differentiated Tem; Th, T helper; Th1*, atypical Th1/Th17; Treg, T regulatory; Tscm, stem cell memory T.

Table 2. Exploratory analyses on reactogenicity of study epitopes*

Patient ID number	HLA-DRB1	Diagnosis	Positive ADNA (history)	Positive ADNA (at time of sampling)	Positive anti-histone antibodies (at time of sampling)	Histone-specific responses		
						Histone-specific T cells	Activation-induced molecules	Cytokine response
1	03:01	SLE	Yes	No	No	Yes	No	Yes
2	11:01	SLE	Yes	No	No	Yes	No	No
3	11:01	SLE	No	No	No	Yes	No	Yes
4	11:01	SLE	Yes	Yes	Yes	Yes	Yes	No
11	11:01	SLE	Yes	No	No	Yes	Yes	Yes
12	03:01	SLE	No	No	No	Yes	Yes	No
13	11:01	SLE	Yes	No	No	Yes	Yes	No
17	03:01	SLE	Yes	No	No	Yes	No	No
22	03:01	SLE	No	No	No	Yes	Yes	Yes
25	03:01	SLE	Yes	Yes	No	Yes	No	Yes
28	03:01	SLE	Yes	No	No	No	No	No
30	03:01	SLE	No	Yes	No	No	Yes	Yes
32	03:01	SLE	Yes	No	No	Yes	Yes	No
47	11:01	HC	No	No	No	No	No	Yes
48	03:01	HC	No	No	No	No	No	No
49	11:01	HC	No	No	No	No	No	No
50	03:01 and 11:01	HC	No	No	No	No	No	No
53	03:01	HC	No	No	No	No	No	No

* Comparison of reactogenicity of histone peptides was performed through distinct assays and clinical phenotypes. Histone-specific T cell responses were defined according to the diagnostic threshold set by receiver operating characteristics curve analysis (Supplementary Figure 5). Activation-induced molecule responses were defined as increased CD69 and OX40 after stimulation with histone peptides compared to negative controls. Cytokine responses were defined as at least a 110% increase in the levels of one or more inflammatory cytokines after stimulation with histone peptides. ADNA, anti-DNA antibody; DRB, DR beta; HC, healthy control; ID, identifier; SLE, systemic lupus erythematosus.

T cells preferentially polarized into T helper (Th) 2-specialized cell subsets. Histone-specific T regulatory (Treg) cells were also expanded (Figure 1F). In the context of quantitatively suppressed in vitro T cell responses to self-antigens (Table 2; Supplementary Figure 6), qualitatively, stimulation with histone synthetic sequences elicited a relatively higher release of IL-5, IL-9, and IL-17F in patients with SLE compared to HCs (Supplementary Figure 7). No differences were observed for other cytokines. In contrast to antigen-specific $CD4^+$ T cell trends, total T_N cells were reduced, total stem cell memory T (T_{SCM}) and effector memory T (T_{EM}) cells increased in patients with SLE in comparison to HCs, and no difference was found in terms of polarization (Table 3), indicating that the characteristics of total T cells in patients with SLE do not apparently reflect those of expanded autoreactive clones in the peripheral blood.

Disease activity and treatments. Histone-specific T cells were lower in patients with active disease (LLDAS = 0). In particular, less histone-specific T_{EM} cells were found in patients with active disease (0.002% $CD3^+$ cells, IQR = 0–0.007%) compared to patients in remission (0.011% $CD3^+$ cells, IQR = 0.004–0.043%, P = 0.036). Histone-specific Th1, Th2, and atypical Th1/Th17 (Th1*) cells were less expanded in patients with active SLE than in patients in remission (Figure 2A–D). Histone-specific Treg cells were also virtually undetectable in the blood of patients with active SLE, and their concentration was inversely correlated with the SLEDAI-2K score (rho = –0.451, P = 0.005; Figure 2E–H). Identical trends were observed at intraindividual comparisons (Figure 3). Histone-specific Treg cells were also inversely correlated with anti-histone antibody concentration (rho = –0.522; P = 0.002). In contrast to autoantigen-specific T cell dynamics, no significant differences in total $CD4^+$ T cell differentiation and polarization among patients with active and quiescent SLE were observed (Table 3).

Patients with SLE who received immunosuppressants had expanded $CD4^+$ histone-specific T_N cells and terminally differentiated T_{EM} (T_{EMRA}) cells. Histone-specific Th2 cells were more represented in patients who did not receive immunosuppressants (33.64%, IQR = 12.66–40.00%) than in patients who received immunosuppressants (9.29%, IQR = 0.95–14.81%; P = 0.010). Patients who received mycophenolate mofetil (12 of 32) had higher total, T_{EM}, T_{EMRA}, and Th1-polarized $CD4^+$ histone-specific cells than patients who did not receive it (Supplementary Figure 8), whereas receiving prednisone had no clear influence on autoreactive T cell homeostasis in the blood, and no detectable differences were observed between patients who did or did not receive other immunosuppressants such as azathioprine or methotrexate (not shown).

DISCUSSION

By analyzing total and HLA-restricted, antigen-specific T cell responses in patients with SLE, HCs, and patients with TAK, we

Table 3. Total T cell distribution*

T cell type	Patients with SLE			Patients with TAK (n = 11)	HCs (n = 10)
	Total (n = 32)	Active (n = 9)	Remission (n = 23)		
$CD4^+$ T cell differentiation, n (%)					
T_N	27.3 (19.7–38.3)	26.2 (14.8–30.6)	29.0 (20.4–41.2)	25.14 (14.3–31.6)[a]	38.5 (30.6–44.7)
T_{SCM}	2.0 (1.2–2.6)[a]	2.3 (1.7–3.3)[b]	1.7 (1.0–2.6)[a]	1.4 (0.7–2.4)	0.9 (0.7–1.8)
T_{CM}	14.5 (11.3–22.2)	15.1 (12.4–22.9)	14.0 (10.8–21.5)	19.3 (13.2–23.0)	14.7 (9.0–25.1)
T_{EM}	23.8 (15.9–32.7)[a]	34.5 (14.5–35.1)[a]	21.9 (16.0–29.2)[a]	21.0 (11.8–31.2)	12.5 (10.3–22.9)
T_{EMRA}	17.0 (11.4–22.3)	15.9 (11.6–16.8)	19.1 (11.3–25.1)	16.3 (11.9–27.5)	25.5 (12.5–28.4)
$CD4^+$ T cell polarization, n (%)					
Th1	6.0 (3.4–7.9)	6.5 (3.7–9.1)	6.0 (3.1–7.0)	5.6 (4.1–9.8)	5.8 (1.9–9.5)
Th2	15.0 (11.4–20.7)	15.5 (12.3–19.5)	14.8 (11.2–21.6)	13.9 (6.5–16.6)	10.1 (6.9–15.4)
Th17	3.0 (1.3–5.8)	2.9 (1.8–5.3)	3.1 (1.1–6.3)	3.6 (2.2–5.3)	2.0 (1.4–4.2)
Th1*	1.3 (0.7–2.1)	1.3 (0.8–2.5)	1.3 (0.6–2.0)	2.7 (0.6–3.6)[a]	1.2 (0.8–2.1)
Treg	1.8 (1.0–3.4)	1.8 (1.5–2.6)	1.7 (0.9–3.6)	1.4 (0.7–4.9)	1.6 (1.2–2.3)
$CD8^+$ T cells differentiation, n (%)					
T_N	28.4 (19.3–41.0)	16.7 (15.7–30.6)	30.0 (19.8–42.8)	15.5 (12.1–44.0)	28.4 (23.8–36.2)
T_{SCM}	3.3 (2.2–5.3)	11.1 (4.7–12.0)[c]	3.2 (2.1–4.1)	6.3 (1.7–11.7)	3.8 (1.6–5.4)
T_{CM}	4.2 (1.8–7.1)	4.1 (1.4–5.2)	4.2 (1.9–7.3)	3.6 (3.0–5.6)	3.4 (2.3–4.1)
T_{EM}	7.8 (4.9–10.8)	8.5 (4.5–9.8)	6.9 (5.0–13.2)	11.5 (6.5–13.0)	4.0 (3.5–11.5)
T_{EMRA}	43.9 (34.1–53.6)	53.7 (40.0–58.0)	42.9 (32.5–49.0)	44.2 (34.2–48.6)	45.3 (32.7–57.8)

* Pairwise nonparametric comparison were performed among all groups. HC, healthy control; SLE, systemic lupus erythematosus; TAK, Takayasu arteritis; T_{CM}, central memory T; T_{EM}, effector memory; T_{EMRA}, terminally differentiated T_{EM}; Th, T helper; Th1*, atypical Th1/Th17; T_N, naive T; Treg, T regulatory; T_{SCM}, stem cell memory T.
[a] $P < 0.05$ versus HCs.
[b] $P < 0.01$ versus HCs.
[c] $P < 0.05$ versus patients with SLE in remission.

found that blood histone-specific T cells are a hallmark of SLE, associate with ADNA immunity, and decrease in number during active disease, independent of treatment and of nonspecific $CD4^+$ T cells characteristics. Histone-specific and, more generally, autoreactive T cells can be observed in healthy subjects.[40,41] However, sustained inflammatory responses to autoantigens are usually dampened by regulatory mechanisms. Consistently, we and others[18,42] found that PBMCs respond to self-peptides by suppressing cell activation and cytokine release. Significantly higher suppression was observed in HCs than in patients with SLE.

Current pathophysiologic models of patients with SLE predicate that extracellular nuclear constituents, released by neutrophil extracellular trap formation, and impaired clearance of debris generated by apoptosis, necroptosis, and other forms of cell death synergize with aberrant release of DNA-associated proteins (including histones and alarmins such as high-mobility group box 1),[6] enhancing natural latent antinuclear B and T cell reactivity[43] toward cellular and humoral autoimmunity.[1,41,44] Loss of tolerance to nucleosomes has been postulated to be one of the seeding events in the development of autoreactive responses to a multitude of antigens in patients with SLE.[45] Peptide epitopes derived from nucleosome major protein constituents, histones, have been identified as the primary antigens recognized by anti-nucleosome T cells.[44,46,47] T cell sensitization to nucleosomes is then followed by the development of antibodies against a variety of nuclear antigens, including histones, native DNA, or the Smith antigen. These in turn can bind a multitude of potential cellular and tissue targets.[48] Among autoantibodies, anti-histone antibodies have been linked to variations in SLE inflammatory status[18] but have a limited diagnostic and disease monitoring role because their restricted clustering in a subset of patients with higher disease activity and their low specificity,[45,49] which is consistent with our findings. Anti-histone antibodies in SLE might be the epiphenomenon of more complex aberrations of the immune response toward chromatin components while contributing little to the pathogenesis of the disease.[18,45,50] ADNAs have instead emerged for their clinical and pathogenic relevance because of their frequency, correlation with disease activity fluctuations,[51] and potential direct involvement in tissue damage.[52–54] Somatic hypermutation is required for functional ADNAs, supporting a nonredundant role of histone-specific Th cells in ADNA-driven pathogenic events.[55]

Previous studies have indeed revealed that $CD4^+$ T cells purified from patients with SLE with positive HLA-DRB1*03:01, endowed with histone peptide-compatible TCR[46] and reacting to histone peptide exposure with proliferation and cytokine release,[19] selectively enhance the production of ADNAs when co-cultured with autologous B cells.[44,56] These processes might also be favored by (a) constitutional alterations in the biology of the TCR[8], and (b) impaired suppression of latent T cell responses to self-antigens.[18] However, no previous study has isolated and

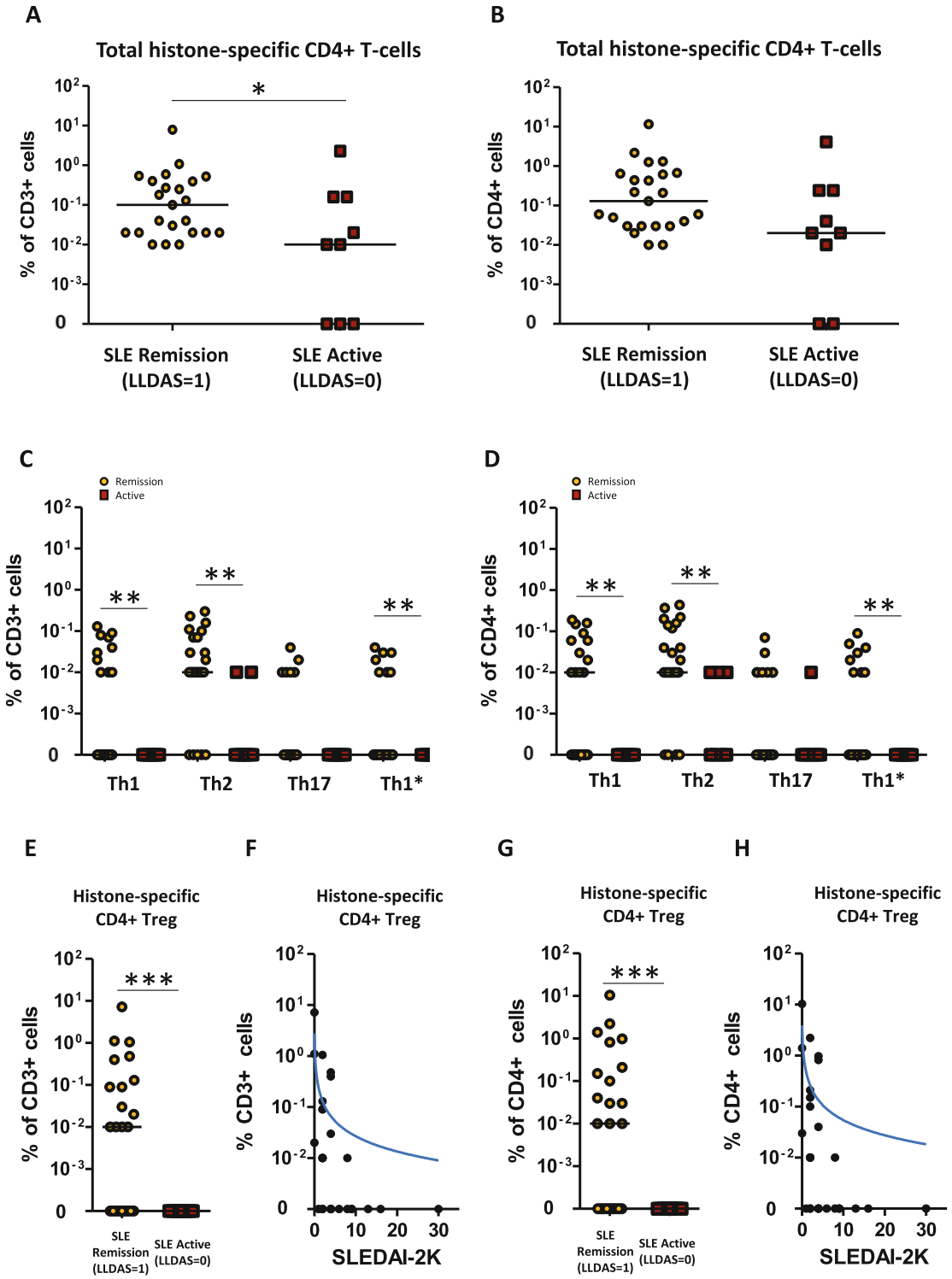

Figure 2. Histone-specific CD4$^+$ T cell variations with activity in patients with SLE. These graphs show the differential expansion of (A and B) total histone-specific CD4$^+$ T cells and (C and D) histone-specific T cell subpopulations by (C and D) effector and (E, F, G, H) regulatory polarization in patients with SLE with distinct degrees of disease activity. (A, C, E, and F) Proportion of histone-specific CD4$^+$ T cells over the total CD3$^+$ cells and (B, D, G, and H) over the total CD4$^+$ cells. (A–E and G) Dot plots depicting comparisons between histone-specific CD4$^+$ T cell frequency between patients in remission (yellow dots) and those with active disease (red squares). *P < 0.05, **P < 0.010, ***P < 0.001, with respect to patients with active disease. (F and H) Scatterplots depicting the inverse correlation between histone-specific Treg and SLEDAI-2K, with blue lines representing interpolation hyperbolic curves for patients with nonnull histone-specific CD4$^+$ T cells (r^2 = 0.355 for the proportion of CD3$^+$ cells, r^2 = 0.351 for the proportion of CD4$^+$ cells). LLDAS, Lupus Low Disease Activity State; SLE, systemic lupus erythematosus; SLEDAI-2K, SLE Disease Activity Index 2000; Th, T helper; Th1*, atypical Th1/Th17; Treg, T regulatory. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42778/abstract.

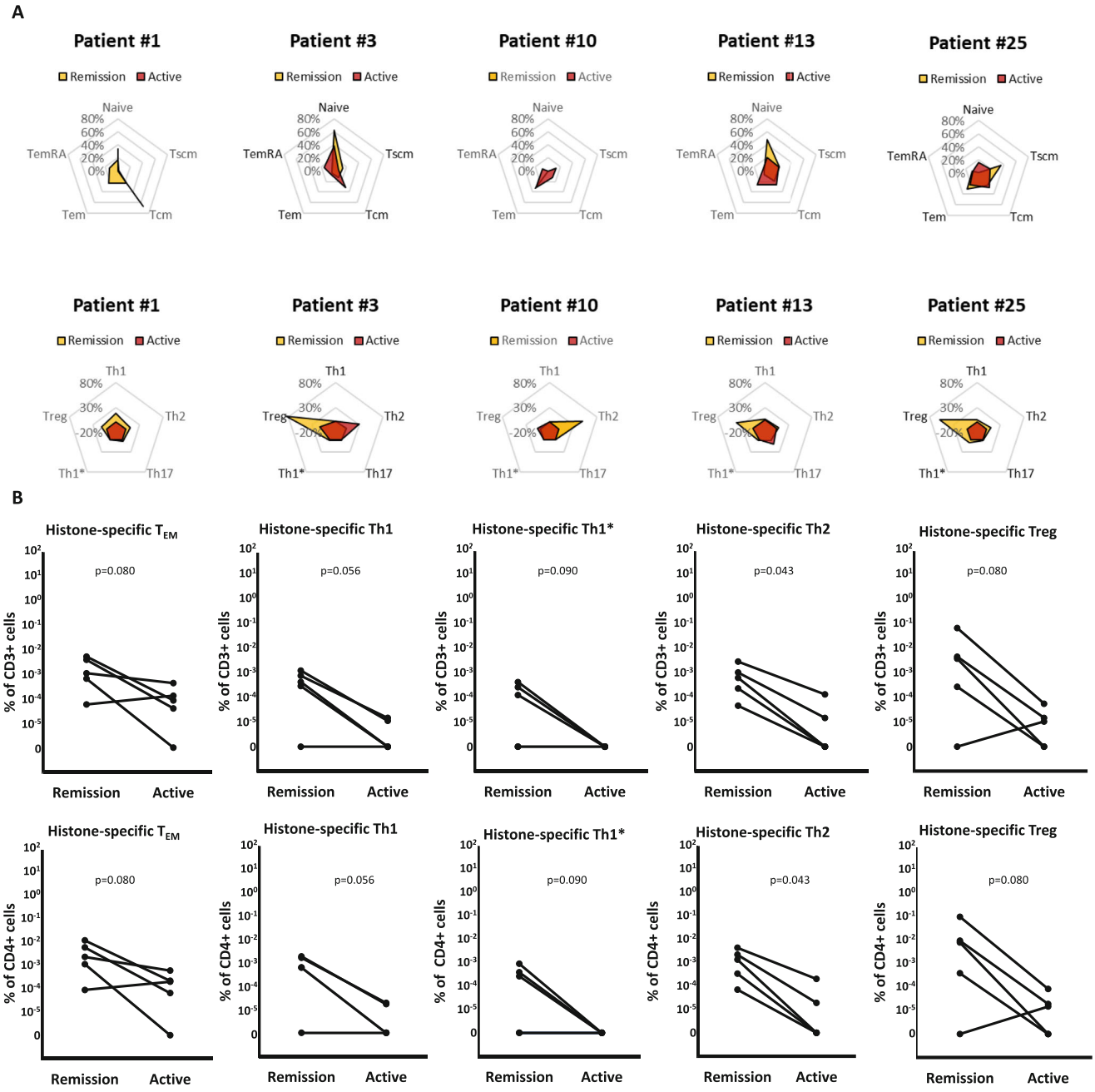

Figure 3. Intraindividual variations in histone-specific CD4$^+$ T cells. Histone-specific CD4$^+$ T cell dynamics were assessed by flow cytometry during active disease and remission in five patients with SLE. (A) Relative proportion of histone-specific T cell subpopulations by differentiation stage (upper subpanels) and polarization (lower subpanels) within the major histocompatibility complex–positive T cell compartment. (B) Variations in selected histone-specific T cell subpopulations (T_{EM}, Th1, Th2, Th1*, and Treg) with respect to CD3$^+$ (upper subpanels) and CD4$^+$ compartments (lower subpanels). Individual patient data are reported in Supplementary Table 3. T_{CM}, central memory T; T_{EM}, effector memory T; T_{EMRA}, terminally differentiated T_{EM}; Th, T helper; Th1*, atypical Th1/Th17; Treg, T regulatory; T_{SCM}, stem cell memory T. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42778/abstract.

functionally characterized histone-specific T cells by selective detection of cells bearing TCR able to effectively engage definite self-peptides bound to autologous HLAs.

Here, for the first time, we have employed peptide-bound HLA tetramers to perform direct ex vivo visualization of T cells able to recognize histone peptides in the context of patients with HLA and found evidence of association with variations in disease activity and humoral immunity against DNA. Our approach was accurate in identifying patients with SLE and with ADNAs and might be less affected by confounders related to experimental conditions and treatments.[17,19,42] Mechanistically, persistence of histone-specific T cells might account for long-term maintenance

of immunologic memory toward nuclear autoantigens. Moreover, variations in the balance between effector and regulatory autoreactive T cells in the blood and peripheral tissues might correlate with the relapsing/remitting course of SLE. Epistemologically, although the association with ADNAs is reassuring for the ability of histone-specific T cells to capture a biologically relevant phenomenon, finding an incomplete overlap between ADNA and histone-specific T cell fluctuations suggests that the latter might not only have a surrogate role toward ADNAs but possibly expand and complement their diagnostic significance. More generally, histone-specific T cell tracking and tackling has the potential to move current diagnostic and therapeutic strategies a step closer to the molecular core of the pathogenesis of the disease.

We observed that the expansion of autoreactive T cells was harmonic within all memory compartments, including T_N and T_{SCM} cells. Antigen-specific T_N cell frequencies correlate with the magnitude of downstream memory responses.[57] Accordingly, detecting relatively high levels of histone-specific T_N cells in patients with SLE may account for long-term renewal of autoreactivity and further support the role of histone-specific T cell in promoting or mediating antigen spread, as observed during the disease course.[58,59] Significant representation of the T_{SCM} cell repertoire within antigen-specific T cells is also consistent with their growingly appreciated role in promoting plasticity and maintenance of the immune response in SLE and other autoimmune diseases.[11,12,60] We observed that decreased autoreactive T_{EM} cells, along with Th1-, Th2-, and Th1*-polarized cells were selectively associated with active disease, whereas variations in the differentiation and polarization of total $CD4^+$ T cell failed to identify clinically relevant clusters of patients with SLE.[9] Of note, intraindividual variations in total antigen-specific T cells mirrored those observed when comparing distinct patients with active and quiescent disease and pointed toward a decreased representation of histone-specific T cells during active disease. Consistently, previous evidence has shown that T_{EM} cells of patients with SLE decrease in blood and become enriched in urine during active lupus nephritis, suggesting that cells recognizing renal antigens actively migrate into target tissues during disease activity.[14] Similar observations have also been replicated in anti-neutrophil cytoplasmic antibody-associated vasculitides.[61] More recently, other authors also consistently reported that autoreactive $CD4^+$ T cells are proportionally higher in the urine of patients with active lupus nephritis compared to blood.[17] Taken together, these data suggest that histone-specific T_{EM} cells might actively participate to the pathogenesis of SLE by redeployment into target tissues in the context of enhanced inflammation.[13,14,17,61]

Histone-specific Treg cell counts were in parallel decreased during active disease and significantly inversely correlated with SLEDAI-2K, supporting the hypothesis that these cells are directly implicated in SLE flares.[13] Conversely, immunosuppression with mycophenolate was associated with higher levels of histone-specific $CD4^+$ T cells, with prominent expansion of terminally differentiated subpopulations. Although supporting a potential anti-inflammatory role of mycophenolate toward T cells in SLE (possibly consisting in the inhibition of antigen-specific cell migration into target tissues[62] besides mycophenolate antiproliferative properties toward B cells), this also indicates that antigen-specific T cell tracking might robustly be employed in SLE diagnostics even in the management of patients with long-lasting disease. Specifically, although complementing clinical judgment in distinguishing SLE from other conditions, histone-specific T cell variations over time might predict the occurrence of flares besides being not affected by ongoing treatments. While TAK constituted an ideal control group for SLE in terms of demographics, morbidity burden, and putative role of T cells, patients with TAK had a slightly higher immunosuppressive load compared to patients with SLE, warranting further research to address the role of immunosuppressants in modulating T cell biology in this disease.

Additional studies are also required to overcome the limitations of this work, which include the lack of extensive longitudinal data and of representative patients of the whole spectrum of SLE clinical variability. Our observations are also limited by having focused on relatively limited number of HLA-DRB1 variants and on autoantigens, preventing further considerations on alternative antigens and epitopes of potential pathogenic relevance for SLE. More generally, our experimental approach excluded nonpeptide and non-HLA–restricted epitopes. Addressing the role of other antigen-specific T cell subpopulations such as follicular Th cells also constitutes a fascinating prospect for future research, especially in light of the prominent expansion of Th2-polarized histone-specific cells in our cohort.[63] In order to minimize the risk of introducing potential confounders because of the in vitro manipulation of cells, we did not employ tetramer-positive cell enrichment techniques. On the other hand, despite the detection of relatively higher frequency of antigen-specific T cells in our study compared to similar works in other autoimmune disorders,[40] this choice could have affected our ability to detect low-frequency events among the rarer antigen-specific T cell subpopulations. Further research is also needed to determine potential similarities and distinctive traits in antigen-specific T cell responses among patients with SLE and other connective tissue diseases.

In summary, we provide the first direct evidence of the use MHC tetramers and flow cytometry to monitor the plastic expansion or contraction of histone-specific T cells with diverging polarization and/or differentiation rather than nonspecific variations in total T cell phenotypes or serological or inflammation markers as tools to stratify patients with SLE. Tracking antigen-specific T cell endotypes might overcome limitations in SLE diagnostics and patient monitoring over time and complement emerging tools for patient stratification based on HLA and advanced B cell response profiling.[64] Furthermore, the identification of traceable antigen-specific T cell profiles correlating with clinical features might disclose clues for developing treatments targeting mechanisms of SLE immune dysfunction at a molecular level.[65]

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ramirez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ramirez, Tassi, Noviello, Bonini, Manfredi.

Acquisition of data. Ramirez, Mazzi, Moroni, Citterio, Zagato, Tombetti, Baldissera, Bozzolo.

Analysis and interpretation of data. Ramirez, Tassi, Noviello, Doglio, Bonini, Dagna, Manfredi.

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