CHARACTERIZATION OF CELLULAR AND HUMORAL AUTOIMMUNE RESPONSES TO HISTONE H1 AND CORE HISTONES IN HUMAN SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

Objective: To address key aspects of anti-histone autoimmunity in systemic lupus erythematosus (SLE), we performed a detailed characterization of cellular and humoral autoreactivity to histone H1 and the four core histones H2A, H2B, H3, H4 in patients with SLE and healthy controls.

Methods: Peripheral blood mononuclear cells of 41 SLE patients and 28 healthy controls were exposed to individual histones and proliferation was measured by [3H]-thymidine incorporation. H1-reactive T-cell clones were drawn by limiting dilution. Cytokines and total IgG in culture supernatants was measured by ELISA, autoantibodies to histones were determined by ELISA and immunoblotting.

Results: Proliferative responses to H1 were more frequent and more pronounced in cell cultures from SLE patients (p<0.002), while among the core histones only the response to H2A was increased in patient cultures (p<0.01). All histones elicited a Th1-like cytokine response both in patients and controls (high IFNγ and TNFα, no IL-4) with H1 inducing the highest levels of TNFα. However, H1 stimulated production of IgG and anti-histone antibodies only in cell cultures derived from SLE patients. H1-specific T-cell clones from patients and controls showed a CD4⁺CD28⁺ phenotype and a Th1 cytokine profile. Anti-histone antibodies were detected in 51% of SLE patients, were primarily directed to H1, H3 and H4 and predominantly of the IgG2 subtype.

Conclusion: Histone H1 constitutes a major B-cell and T-cell autoantigen in SLE triggering a proinflammatory Th1 response and driving autoantibody production. This suggests that histone H1 may be of considerable relevance for the pathogenesis of SLE.
Antinuclear autoantibodies are a hallmark of SLE. The vast majority of SLE patients develop autoantibodies (autoAbs) against chromatin components which may be directed against single structures or against the complete nucleosome. Among these autoAbs, IgG Abs to dsDNA are a pathognomonic feature of SLE and can be directly pathogenic. [1] However, dsDNA may not be the first target of the autoAb response; rather, autoAbs to other chromatin components, and to histones in particular, may precede anti-dsDNA Ab formation. [2]

Anti-histone Abs can be directed to single histones, to histone-DNA complexes, or to complexes of histones. [3,4] In particular, H2A-H2B dimers frequently elicit humoral anti-histone responses, especially in drug-induced LE. [5,6] Anti-histone Abs can be found in a variety of diseases, including inflammatory hepatic, malignant, infectious and, of course, rheumatic autoimmune diseases. [7-9] They may be present in up to 75% of SLE sera, while their prevalence in other rheumatic diseases usually does not exceed 20%. [10,11] Like anti-dsDNA Abs, also anti-histone Abs correlate with SLE disease activity, [12,13] particularly Abs to histone H1 which appear to be more specific for SLE and may better correlate with disease activity than Abs to core histones. [10] Of note, it is anti-H1 Abs that are responsible for the LE cell phenomenon, which was originally included into the ACR classification criteria for SLE. [14,15]

AutoAbs to chromatin components show immunoglobulin class switching and affinity maturation which are typical features of a T-cell dependent Ag-driven immune response. [16-18] Histones are considered the major T-cell antigens (Ags) in SLE for the generation of autoimmune responses against chromatin components: thus, histone-specific T-cell clones augmented the production of anti-dsDNA, anti-ssDNA and anti-histone Abs in murine models of SLE [19] and also promoted anti-dsDNA production by autologous B-cells in human SLE; [3,20] however, the details of the histone-specific T-cell response in human SLE have not been fully elucidated. In particular, it is not clear which histone constitutes the major autoAg in T-cell activation.

Considering current Ag-specific therapeutic approaches, [21] and in order to specify the impact of individual histones in T-cell activation, we performed a detailed analysis of the cellular and humoral responses against histone H1 and the core histones H2A, H2B, H3 and H4 in SLE patients and healthy controls. The results suggest that histone H1 may indeed be the most important single histone autoAg, both at the B- and T-cell level.
PATIENTS AND METHODS

Patients and controls

Forty one patients with SLE fulfilling the ACR criteria for SLE, [14] and 28 healthy individuals were analyzed; demographic details of patients and controls are given in Suppl. Table 1. In the course of routine blood testing, 40 ml of peripheral blood were drawn after obtaining informed consent from each patient. Disease activity was measured using the SLE disease activity index (SLEDAI), the European Consensus lupus activity measurement (ECLAM), and the SLE activity index score (SIS). [22-25]

T-cell stimulation assays

Peripheral blood mononuclear cells (PBMC) were cultured for 5 days in triplicates in the presence of the Ags (10^5 cells/well) as described. [26] During the last 16h of culture, 0.5 µCi/well [3H]TdR (Amersham Biosciences, Freiburg, Germany) was added, and the incorporated radioactivity was measured by scintillation counting and expressed as counts per minute (cpm). Results are given as stimulation index (SI) defined by the ratio of (mean cpm obtained in cultures incubated with Ag):(mean cpm obtained in cultures incubated in the absence of Ag). An SI ≥2 was regarded a positive response, if, at the same time, the ∆cpm ([mean cpm obtained in cultures with Ag] minus [mean cpm obtained in cultures without Ag]) was >1000 cpm. [3,26,27]

Antigens

Highly purified calf thymus histones H1, H2A, H2B, H3, H4 (Roche Diagnostic GmbH, Mannheim, Germany) were used throughout this study. Purity was confirmed by SDS-PAGE and Coomassie Blue staining. For each SLE patient and healthy control (HC), the individual reactivity of PBMC to histone Ags was tested at 4 different concentrations (i.e. 0.7, 1.25, 2.5 and 5 µg/ml). The concentration yielding the highest SI was used in subsequent experiments (Suppl. Fig. 1). In the majority of patients and HC 2.5 or 5 µg/ml were found optimum concentrations. The control Ag tetanus toxoid (TT) was obtained from Pasteur Merieux Connaught (Willowdale, Ontario, Canada) and used at a concentration of 0.5 U/ml as described (26,28). When indicated, PBMC cultures were also stimulated with 1 µg/ml pokeweed mitogen (PWM; Sigma Aldrich, Vienna, Austria), and/or with 1 µg/ml anti-CD3 monoclonal Ab (R+D Systems, Minneapolis, MN). In some experiments the autoAg hnRNP-A2 was used as an unrelated basic control-Ag (pI=9.3) at 0.35 µg/ml as described (Suppl. Fig. 2). [29]

Detection of cytokines

ELISA assays for IFNγ and IL-4 were obtained from Endogen (Woburn, MA), the ELISA for TNFα was from Biosource (Nivelles, Belgium). Detection limits were 9 pg/ml for IFN-γ, 4 pg/ml for IL-4, and 15 pg/ml for TNFα, respectively. If not stated otherwise, cytokines were measured in SN after 24h of incubation as described. [26,30]
T-cell lines and clones

H1-specific T-cell lines were generated using a previously established protocol. [26,30,31] In brief, 2x10^6 PBMC were stimulated with histone H1 for 5 days in 24-well plates. On day 5, 20 U/ml IL-2 was added, and the culture was continued for 7 days. To generate T cell clones (TCC), T-cell lines were re-stimulated with histone H1 and, after 2 more days, viable T-cell blasts were separated and seeded in limiting dilution (0.5 cells/well) in 96 well plates together with 1x10^5 irradiated allogeneic PBMC as feeder cells. Growing microcultures were then expanded at weekly intervals in the presence of feeder cells and IL-2.

The specificity of TCC was assessed in proliferation assays (as detailed above): 2x10^4 T-cell blasts were stimulated with H1, H2A, H2B, H4 and TT Ags, respectively, in the presence of 10^5 autologous irradiated PBMC. For surface phenotyping, fluorescence-labelled monoclonal Abs against CD3, CD4, CD8, CD28, TCR α/β, TCRγδ (BD Biosciences, Palo Alto, CA) were used. Isotype-matched Abs to irrelevant Ags (Dako, Glostrup, Denmark, or Becton Dickinson) served as negative controls.

Detection of autoantibodies

Serologic reactivities to single histones were determined by immunoblotting as described [32] employing highly purified histones which were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blotted histones were incubated for 40 min with sera diluted 1:100 and bound antibodies were visualized using an alkaline-phosphatase conjugated secondary Ab. Affinity-purified rabbit Abs specific for the individual histones (Upstate) served as positive controls and were later replaced by a human serum containing autoAbs to all 5 histones. For a subset of sera, the IgG subclasses of anti-histone Abs were analyzed by immunoblotting using alkaline-phosphatase conjugated secondary antibodies specific for IgG1, IgG2, IgG3 and IgG4, respectively (The Binding Site, Birmingham, UK).

Levels of total anti-histone and anti-chromatin Abs in serum and culture SN were measured by ELISA (Inova Diagnostics, San Diego, USA). Sensitivity and specificity of both ELISAs was determined by measuring Ab levels in 420 sera from our serum bank (Suppl. Table 2). Results are given as arbitrary units/ml (U/ml) employing the reference sera provided by the manufacturer. Since sera were diluted 1:100 and culture SN 1:10, 10 U/ml in SN would correspond to 1 U/ml in serum. Anti-dsDNA Abs were measured by radioimmunoassay (Ortho-Clinical Diagnostics Inc., Rochester, USA), total IgG in culture SN was measured by ELISA (Alpco Diagnostics, Windham, USA).

T-cell/B-cell interactions and formation of anti-histone autoantibodies

PBMC (10^5 cells/well) obtained from patients with SLE, rheumatoid arthritis (RA) and HC were cultured in the presence or absence of histone H1 as described above. Unspecific polyclonal stimuli such as PWM and anti-CD3 monoclonal Ab served as positive controls. After 7 days of culture, [33] the SN were analyzed for total IgG and anti-histone Abs by ELISA.
**Statistical analysis**

All group results are expressed as mean±SD, if not stated otherwise. Student's t-test, or Fisher's exact test (2-tailed) were used for the comparison of group values and discriminatory parameters, where appropriate. If logarithmic transformation resulted in Gaussian distributions, such transformation was performed. Welch's correction was applied if variances were significantly different. Where appropriate, p values corrected by Bonferroni's method are shown. Pearson and Spearman correlation coefficients were calculated for variables following and not following Gaussian distributions, respectively.

**RESULTS**

**Increased proliferative response to histones H1 and H2A in SLE patients**

Among 41 SLE patients analyzed for cellular reactivity to single histones, 18 (44%) showed a distinct T-cell response (i.e. SI>2) to histone H1, as compared to only 3 responders (11%) among HC (p<0.01, Fig. 1A), and responses of SLE patients were significantly higher than those of healthy individuals (p<0.002, Table 1).

All four core histones elicited responses in PBMC of both SLE patients and HC, but the frequency of responders was similar in both groups (Fig. 1B) and significant group differences between patients and HC were seen only for H2A (p<0.01, Table 1). Interestingly, in SLE patients, but not in controls, H1 responses correlated significantly with responses to the core histones H2A, H2B, and H4 (Table 1).
Table 1. Cellular proliferation and cytokine production of PBMC in response to histone antigens.

<table>
<thead>
<tr>
<th></th>
<th>H1</th>
<th>H2A</th>
<th>H2B</th>
<th>H3</th>
<th>H4</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SLE</strong> (n=41)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pos. response</td>
<td>44%*</td>
<td>50%</td>
<td>60%</td>
<td>26%</td>
<td>23%</td>
<td>66%</td>
</tr>
<tr>
<td>SI</td>
<td>2.3±1.5*</td>
<td>2.9±2.4*</td>
<td>2.8±1.7</td>
<td>1.9±1.7</td>
<td>1.5±0.8</td>
<td>4.9±5.3</td>
</tr>
<tr>
<td>cpm</td>
<td>7466</td>
<td>3746</td>
<td>4549</td>
<td>4133</td>
<td>2556</td>
<td>5951</td>
</tr>
<tr>
<td>(bgd:1535±1326)</td>
<td>±2815</td>
<td>±3240</td>
<td>±4745</td>
<td>±7811</td>
<td>±2571</td>
<td>±7609</td>
</tr>
<tr>
<td>correlation with H1 response</td>
<td>p&lt;0.001</td>
<td>p=0.01</td>
<td>p= n.s.</td>
<td>p&lt;0.01</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td><strong>HC</strong> (n=28)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pos. response</td>
<td>11%*</td>
<td>26%</td>
<td>37%</td>
<td>26%</td>
<td>21%</td>
<td>85%</td>
</tr>
<tr>
<td>SI</td>
<td>1.5±0.4*</td>
<td>1.5±0.8*</td>
<td>2.2±1.6</td>
<td>1.7±1.2</td>
<td>1.4±0.7</td>
<td>4.7±4.3</td>
</tr>
<tr>
<td>cpm</td>
<td>4161±</td>
<td>4536±</td>
<td>5810±</td>
<td>4071±</td>
<td>4030±</td>
<td>12590±</td>
</tr>
<tr>
<td>(bgd. 2842±2441)</td>
<td>3413</td>
<td>4338</td>
<td>4537</td>
<td>2887</td>
<td>3371</td>
<td>9478</td>
</tr>
<tr>
<td>correlation with H1 response</td>
<td>p= n.s.</td>
<td>p= n.s.</td>
<td>p= n.s.</td>
<td>p&lt;0.05</td>
<td>p= n.s.</td>
<td>p= n.s.</td>
</tr>
<tr>
<td><strong>Cytokine production</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SLE</strong></td>
<td>IFNγ</td>
<td>69±50</td>
<td>20±7</td>
<td>56±25</td>
<td>17±9</td>
<td>41±25</td>
</tr>
<tr>
<td>[pg/ml, mean±SEM]</td>
<td>66±25</td>
<td>50±38</td>
<td>40±0.3</td>
<td>11±6</td>
<td>39±35</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>HC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[pg/ml, mean±SEM]</td>
<td>68±33</td>
<td>25±9</td>
<td>53±20</td>
<td>16±7</td>
<td>41±20</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>SLE+HC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>630±135</td>
<td>259±108</td>
<td>526±141</td>
<td>190±67</td>
<td>276±109</td>
<td>n.d.</td>
</tr>
<tr>
<td>TNFα</td>
<td>912±192</td>
<td>263±31</td>
<td>457±75</td>
<td>248±107</td>
<td>339±151</td>
<td>n.d.</td>
</tr>
<tr>
<td>[pg/ml, mean±SEM]</td>
<td>734±112</td>
<td>260±80</td>
<td>510±109</td>
<td>200±54</td>
<td>283±88</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

In SLE patients cellular reactivity to H1 and H2A was significantly elevated and H1 responses correlated with responses to H2A, H2B, and H4. Similar amounts of IFNγ and TNFα were secreted by PBMC of SLE patients and HC but highest TNFα levels were observed after stimulation with H1.

*denotes a significant difference (p<0.01) in cellular reactivity between SLE patients and HC. # denotes a significant difference versus H1-induced IFNγ and TNFα production.

Values are given as mean±SD, if not stated otherwise.

Abbreviations: TT=tetanus toxoid, bgd=background proliferation without Ag, n.d.=not done.
IFNγ and TNFα, but not IL-4 is secreted in response to histone Ags

The cytokines IFNγ, TNFα and IL-4 were measured in SN of SLE and control PBMC after stimulation with H1, H2A, H2B, H3, and H4. All 5 histones elicited production of IFNγ and TNFα, while IL-4 was not detectable (Table 1). Cytokine levels in SN of SLE patients and HC showed a similar pattern, with the highest values measured upon stimulation with H1. Remarkably, TNFα levels exceeded IFNγ levels by about ten-fold, and TNFα production induced by H1 was significantly higher than the production elicited by H2A, H3, or H4 (Table 1).

H1-specific T-cell clones display a Th1 phenotype

Seven H1-specific TCC could be generated from one SLE patient and two HC (Table 2). These clones did not cross-react with other histones or TT (Fig. 2 and Suppl. Fig. 3). All TCC expressed CD3 and were of CD4⁺CD8⁻TCRαβ⁺ phenotype (Table 2). Similar to PBMC in the primary culture, all clones produced high amounts of IFNγ, with SN levels exceeding 3000 pg/ml in 6 of 7 TCC, whereas IL-4 was not detectable. Furthermore, all clones secreted TNFα with particularly high levels produced by the SLE patient derived TCC (Table 2). Thus, all H1-specific TCC displayed features compatible with a proinflammatory Th1 phenotype.

**Table 2. Phenotyping of histone H1 specific T-cell clones.**

<table>
<thead>
<tr>
<th>TCC</th>
<th>Diagnosis</th>
<th>Surface Phenotyping</th>
<th>SI</th>
<th>H1 cpm</th>
<th>bgd cpm</th>
<th>IFNγ pg/ml</th>
<th>IL-4 pg/ml</th>
<th>TNFα pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA04</td>
<td>SLE</td>
<td>CD4⁺CD8⁻CD28⁺</td>
<td>2.5</td>
<td>1638</td>
<td>655</td>
<td>&gt; 3000</td>
<td>&lt; 4</td>
<td>&gt;1385</td>
</tr>
<tr>
<td>TA08</td>
<td>SLE</td>
<td>CD4⁺CD8⁻CD28⁺</td>
<td>2.3</td>
<td>1730</td>
<td>752</td>
<td>&gt; 3000</td>
<td>&lt; 4</td>
<td>&gt;1385</td>
</tr>
<tr>
<td>TA30</td>
<td>SLE</td>
<td>CD4⁺CD8⁻CD28⁺</td>
<td>4.4</td>
<td>1688</td>
<td>379</td>
<td>&gt; 3000</td>
<td>&lt; 4</td>
<td>&gt;1385</td>
</tr>
<tr>
<td>TA89</td>
<td>SLE</td>
<td>CD4⁺CD8⁻CD28⁺</td>
<td>2.5</td>
<td>1245</td>
<td>498</td>
<td>&gt; 3000</td>
<td>&lt; 4</td>
<td>n.d.</td>
</tr>
<tr>
<td>RI89</td>
<td>HC</td>
<td>CD4⁺CD8⁻CD28⁺</td>
<td>2.0</td>
<td>12794</td>
<td>6397</td>
<td>339</td>
<td>&lt; 4</td>
<td>538</td>
</tr>
<tr>
<td>SC04</td>
<td>HC</td>
<td>CD4⁺CD8⁻CD28⁺</td>
<td>11.5</td>
<td>6820</td>
<td>593</td>
<td>&gt; 3000</td>
<td>&lt; 4</td>
<td>496</td>
</tr>
<tr>
<td>SC104</td>
<td>HC</td>
<td>CD4⁺CD8⁻CD28⁺</td>
<td>9.7</td>
<td>5810</td>
<td>599</td>
<td>&gt; 3000</td>
<td>&lt; 4</td>
<td>478</td>
</tr>
</tbody>
</table>

Seven TCC specific for H1 were established from one SLE patient (n=4) and two HC (n=3). All TCC showed a CD4⁺CD8⁻CD28⁺ phenotype, expressed T-cell receptor (TCR) αβ and produced high amounts of IFNγ and TNFα but no IL-4.
**Anti-histone antibodies are predominantly of the IgG2 subclass and mainly directed to histones H1, H3 and H4**

By ELISA autoAbs to total histones were detected in 41% of SLE sera, but not in sera of HC (p<0.001). Anti-histone Abs correlated with anti-chromatin Abs (r=0.75, p<0.0001), which were present in 41% of SLE sera, and anti-dsDNA Abs (r=0.54, p<0.001), but not with total IgG levels. In addition, there was a significant correlation between anti-histone Abs and disease activity for all 3 activity scores (for SIS: r=38, p<0.05).

When reactivities to individual histones were analyzed by immunoblotting all positive ELISA results were confirmed. In addition, 4 SLE patients and 2 HC who had been negative by ELISA were positive in the immunoblot assay; of note, all 4 SLE sera contained Abs to dsDNA and one serum was additionally positive in the anti-chromatin assay, in contrast to the two HC sera. Thus, 51% of SLE sera and 7% of HC sera showed anti-histone reactivity on immunoblots. Almost all positive SLE sera were reactive with H1, H3 and H4, while reactions to H2A and H2B occurred less frequently (Fig. 3). In all but one of 12 SLE sera selected for IgG subclass analysis anti-histone Abs were of the IgG2 type, while IgG1 anti-histone Abs were detected in only one serum (not shown) further supporting the concept of a Th1-driven autoimmune response.

**Association between T-cell and B-cell responses: histone H1 induces anti-histone Ab production in PBMC of SLE patients**

In SLE patients, the presence of anti-H1 Abs was linked to increased cellular reactivity to H1: thus, 75% of patients showing increased T-cell responsiveness to H1 had anti-H1 Abs as compared to 38% of non-responders (p<0.05). In contrast, such an association was not seen for the core histones, including H2A. We therefore sought to investigate the effect of H1-mediated T-cell activation on IgG production and autoAb formation by measuring total IgG and anti-histone Abs in the SN of PBMC from patients with SLE or RA and HC after 7 days of cultivation (Fig. 4). Baseline IgG levels were similar in all cultures and polyclonal stimulation by anti-CD3 and PWM induced only small increases of IgG which were most pronounced in SLE cultures (p=n.s.). However, IgG levels increased upon stimulation with H1 only in SLE cultures and levels were significantly higher (p=0.02) than in H1-stimulated cultures of HC (Fig. 4A). Baseline levels of anti-histone Abs were similar in SLE and RA cultures and at the lower detection limit in HC cultures. Stimulation with H1 increased anti-histone Ab levels above baseline again only in SLE cultures (p<0.04) resulting in a significant difference (p=0.02) when compared to H1-stimulated PBMC of HC (Fig. 4B). Of note, all SLE patients had serum Abs to histones and/or dsDNA, in contrast to RA patients and HC. The control Ag TT stimulated IgG production to a similar extent as anti-CD3+PWM but had no effect on production of anti-histone Abs.
DISCUSSION

Anti-nucleosomal autoimmune responses seem to play a pivotal role in the pathogenesis of SLE, but it is still unclear which histone(s) form the primary target(s) for histone-specific autoreactive T-cells. To address this question, we performed a detailed investigation on autoimmune reactions to all 5 histones at the T-cell and additionally also at the B-cell level.

All histone Ags elicited proliferative responses in PBMC cultures from both SLE patients and HC, but significant differences were found only for H1 and H2A. Interestingly, cellular reactivity to H1 correlated significantly with cellular responses to histones H2A, H2B and H4 suggesting that H1-specific T-cells may at least partially drive immune responses to other histone Ags, presumably due to the mechanism of intermolecular epitope spreading as has been described for spliceosomal autoAgs in SLE. [34]

In line with previous observations, histones H1, H3 and H4 appeared to be major Ags of the humoral response in SLE, while Abs to H2A and H2B were less frequently found. [10] However, concerning these two histones, H2A-H2B dimers may rather form the preferred target of autoAbs, especially in drug-induced LE, [5, 6] an issue that was not addressed in our study. It is interesting that, despite the relatively high prevalence of autoAbs to H3 and H4 in sera of SLE patients, cellular reactivity to these histones was not different from healthy controls. Moreover, TNFα secretion elicited by H3 and H4 in the primary culture assays was considerably lower than TNFα secretion induced by H1. Thus, it is possible that the autoimmune response to H3 and H4 may have less pathogenic consequences, or might even exert protective functions. This assumption is supported by observations made in experimental lupus in which peptides derived from H3 or H4, in contrast to H1-specific peptides, suppressed or delayed lupus symptoms when applied to lupus-prone mice. [35,36]

The data obtained in our system of cultured PBMC suggest that H1-specific T-cell help for autologous B cells led to the observed increases in anti-histone Ab levels which were seen only in SLE cultures, but in cultures from RA patients or HC. Several reports underline the ability of histone-specific Th cells to trigger the production of autoAbs (such as anti-dsDNA), both in human and murine SLE. [3,37] Although major T- and B-cell epitopes have been reported not only in H1 (but also in H2B, H3, H4), H1 seems particularly important in promoting lupus-like features in mice. Thus, a peptide derived from histone H1 (H122-42) stimulated autoimmune Th cells, which, in turn, augmented the production of pathogenic antinuclear Abs, and this H1 peptide was more potent than core histones in accelerating nephritis in lupus-prone mice. [19,37,38]

The occurrence of autoreactive T-cells in healthy individuals is in line with previous investigations on potent autoAgs [3,36,39-41] and further supports the idea that also peripheral tolerance mechanisms are disturbed in SLE. [42] In contrast to other reports, [29,43,44] no phenotypic differences were seen between TCC derived from SLE patients and HC including expression of cell surface markers and cytokine profile. Although they were derived from only one patient and two HC, it is noteworthy that they were highly specific for H1 and closely mirrored the cytokine pattern observed in PBMC after H1-stimulation with pronounced production of IFNγ and TNFα. Thus, these clones may at least partially reflect the complex pathological situation in SLE.

Some authors have, mainly based on serum cytokine levels, considered SLE a Th2-mediated disease, [45] but the issue has remained controversial since other studies reported elevated
Th1 cytokines, [46] or a balanced intracellular Th1/Th2 ratio in peripheral SLE lymphocytes. [47] In a subgroup of patients with severe renal SLE, however, the intracellular cytokine ratio was shifted towards a Th1 phenotype, which was also reflected by histopathological features in the inflamed kidneys. [47,48] Experiments in murine lupus support these findings: A shift towards Th1 predominance was associated with disease acceleration, [49] and lupus-like disease after immunization with a peptide mimotope of dsDNA was Th1-mediated. [50]

Interestingly, H1 stimulation did not only induce IFNγ but also TNFα production which exceeded IFNγ production several-fold. Since TNFα correlates with SLE disease activity, [51] and since its therapeutical blockade results in clinical benefit, including amelioration of lupus nephritis, [52] it is tempting to speculate that histone specific T-cells, and particularly those directed to H1, could be important stimulators of inflammation in SLE. This further supports the concept of a histone-driven Th1 (or Th0) response both in human and murine SLE, [3,38] which is in accordance with our observation that anti-histone Abs were predominantly of the IgG2 subclass.

In view of all these data, we conclude that histone H1 constitutes an important B- and T-cell autoAg of probable pathogenic relevance in SLE. Thus, in search of Ag-specific treatment strategies the H1-directed autoimmune response may well constitute a relevant target.

ACKNOWLEDGEMENTS

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**FIGURE LEGENDS**

**Figure 1. Proliferative responses to histone H1 and core histones in SLE patients and healthy controls.**

PBMC from 41 SLE patients and 28 healthy control persons were cultured in the presence or absence of histone Ags and proliferation was measured by [³H]thymidine incorporation. A positive response was defined as SI>2 and Δcpm>1000 cpm. Results obtained at the optimum histone concentration are shown which was determined for each histone in all patients and HC as detailed in the Methods section; typical dose-response curves are shown in Suppl. Fig 1. For SLE patients, the proliferative response ranged from 1535±1326 cpm (mean±SD) in the absence of any antigen to 7466±2815 cpm after H1-stimulation to 21540±22830 cpm after polyclonal stimulation with PHA.

(A) Response to histone H1. A positive response was seen in 44% of SLE patients as compared to 11% of HC (p<0.01) and the strength of the proliferative response was significantly higher in SLE cultures (mean SI±SD: 2.3±1.5 vs. 1.5±0.4, p<0.002).

(B) Response to core histones. Frequencies of responders did not differ between SLE (black triangles) and HC (white triangles) and a significant difference in the mean SI was observed only for H2A (mean SI: 2.9±2.4 vs. 1.5±0.8, p<0.01).

**Figure 2. H1-specific T-cell clones.**

Seven TCC specific for H1 could be raised by limiting dilution. Four TCC were generated from one SLE patient (A) and three from two healthy individuals (B). The clones were re-exposed to H1, H2A, H2B, H4 Ags and to TT as a control Ag. All clones showed proliferative responses against H1, but not against core histones or the control Ag TT. Further details on proliferation, surface phenotype and cytokine profile of each individual TCC are presented in Table 2 and Suppl. Fig. 3.

**Figure 3. Immunoblot analysis of anti-histone autoantibodies.**

All sera of SLE patients and HC were investigated by immunoblotting for the presence of autoAbs to individual histones. Representative immunoblots of 32 SLE patients (A) and 8 HC (B) are shown. A human serum reactive with all 5 histones was used as reference (R). Sera considered positive are marked by an asterix. Of note, SLE sera 3, 18, 23 and HC serum 5 showed a negative result in the anti-histone ELISA, all other immunoblot-positive sera were also positive by ELISA. Patients with anti-H1 Abs were also reactive with histones H3 and/or H4 (prevalence 51% and 49%, respectively), while reactivities to H2A and H2B appeared weaker in general (prevalence 35% and 38%, respectively).
Figure 4. Induction of anti-histone Ab production by histone H1 in PBMC cultures of SLE patients.

PBMC from 8 SLE patients, 6 RA patients and 6 HC were cultured in the presence of histone H1, or the polyclonal stimuli anti-CD3 and PWM, or the control Ag TT. After seven days, total IgG and anti-histone Abs were measured in the SN by ELISA. All SLE patients had serum Abs to histones and/or dsDNA, in contrast to controls subjects.

(A) Induction of IgG production. IgG baseline levels were similar in all cultures and moderate but insignificant increases were observed upon stimulation with anti-CD3+PWM or in the presence of TT. Upon exposure to histone H1, IgG production increased in SLE patient derived cultures (#, p=0.02 as compared to HC cultures), whereas IgG levels in cultures derived from RA patients or HC did not change.

(B) Induction of anti-histone Ab production. Baseline levels were similar in SLE and RA cultures and very low in HC cultures. Only in SLE cultures histone H1 induced a significant increase of anti-histone Ab levels from baseline (*, p<0.04) and H1-induced anti-histone Ab levels were significantly higher than in H1-stimulated HC cultures (#, p=0.02).
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Dose-response curves for individual histones.
For all patients and controls, the five histone Ags (i.e. H1, H2A, H2B, H3, H4) were tested at 4 different concentrations (i.e. 0.7, 1.25, 2.5 and 5 µg/ml) in primary cultures in order to determine the optimum concentration which was then used in all subsequent experiments. Dose-response curves obtained with PBMC of two representative SLE patients are shown. In general, 2.5 or 5 µg/ml was found to be the optimum concentration, in some cases lower concentrations yielded better results (e.g. 1.25 µg/ml for H1 as shown in panel A).
H1 stimulation is indicated by a black diamond, core histone stimulation by open symbols as indicated within the Figure.

Supplementary Figure 2. Proliferative responses to histones and the basic control Ag hnRNP-A2.
In a smaller series of experiments, freshly drawn PBMC from 7 SLE patients, 5 RA patients and 5 HC were stimulated with H1, the core histones anti-CD3+PWM (as positive control) as well as with the basic protein hnRNP-A2, a known autoantigen in SLE and RA. [26, 29] Among the 7 SLE patients, one responded to hnRNP-A2 but not to any of the histone Ags (A), one exclusively to H1 (B), while two patients showed a positive response to H1 and other histones (H2B and/or H4) but not to hnRNP-A2. Among the control subjects, one RA patient responded to H1, H2A, H4 and hnRNP-A2 and one HC exclusively to H1. Thus, the cellular autoimmune responses to histone Ags and anti-hnRNP-A2 appeared to occur independently.

Supplementary Figure 3. Proliferative responses of histone H1-specific TCC from SLE patients and HC.
Proliferative responses of H1-specific TCC derived from SLE patients (A) and healthy controls (B) following re-exposure to histone Ags H1, H2A, H2B, H4 and TT as a control Ag are shown. As can be clearly seen, all clones proliferated (SI>2) in response to stimulation with H1 (mean SI 3.8±2.7), but not against core histones or TT.
Figure 1

A

B

p<0.002

p<0.01

p=n.s.
Figure 2

A. SLE

B. HC
Figure 3

A. SLE

B. HC
Figure 4

A

![Graph showing IgG levels in SLE, RA, and HC groups.](image)

B

![Graph showing anti-histone Abs levels in SLE, RA, and HC groups.](image)
### Supplementary Table 1. Characteristics of SLE patients and HC.

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>HC</th>
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<tbody>
<tr>
<td>number of persons</td>
<td>n</td>
<td>41</td>
</tr>
<tr>
<td>female</td>
<td>n (%)</td>
<td>39 (95)</td>
</tr>
<tr>
<td>age (years)</td>
<td></td>
<td>41±13#</td>
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<tr>
<td>disease duration (months)</td>
<td></td>
<td>127±107</td>
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<tr>
<td>SIS/SLEDAI/ECLAM</td>
<td></td>
<td>5±3/5±5/2±2</td>
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<tr>
<td>anti-histone Abs</td>
<td></td>
<td></td>
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<tr>
<td>immunoblot % positive</td>
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<td>51</td>
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<tr>
<td>ELISA % positive</td>
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<td>41</td>
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<tr>
<td>anti-dsDNA Abs % positive</td>
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<td>69%</td>
</tr>
<tr>
<td>IU/ml</td>
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<td>38±60</td>
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<tr>
<td>Antinuclear Abs n (%)</td>
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<td>organ involvement n (%)</td>
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</tr>
<tr>
<td>renal</td>
<td></td>
<td>16 (40%)</td>
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<tr>
<td>pneumonitis</td>
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<tr>
<td>cerebral</td>
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<td>15 (38%)</td>
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<td>therapy</td>
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<tr>
<td>glucocorticoids n (%)</td>
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<td>azathioprine * n (%)</td>
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<tr>
<td>chloroquine * n (%)</td>
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<td>13 (33%)</td>
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<tr>
<td>methotrexate n (%)</td>
<td></td>
<td>5 (13%)</td>
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</table>

# group data are given as mean values ± SD

* 3 patients had azathioprine and chloroquine in combination
Supplementary Table 2. Sensitivity, specificity and positive predictive value (PPV) of anti-histone and anti-chromatin (nucleosome) Abs.

<table>
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<tr>
<th>Assay</th>
<th>Histone</th>
<th>Chromatin</th>
<th>Histone</th>
<th>Chromatin</th>
<th>dsDNA</th>
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<tbody>
<tr>
<td>Cut off level</td>
<td>20 U/ml</td>
<td>20 U/ml</td>
<td>30 U/ml</td>
<td>30 U/ml</td>
<td>1:20</td>
</tr>
<tr>
<td>SLE (n=72)</td>
<td>42</td>
<td>35</td>
<td>28</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Controls (n=348)</td>
<td>31</td>
<td>19</td>
<td>9</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

Sensitivity 58.3% 48.6% 38.9% 40.3% 40.3%
Specificity 91.1% 94.5% 97.4% 96.0% 99.1%
PPV 57.5% 63.6% 75.7% 67.4% 90.6%

Abs to total histones (H1 and core histones) and H1-stripped chromatin (nucleosomes) were measured by ELISA (Inova, San Diego, USA), anti-dsDNA Abs were measured by indirect immunofluorescence on Crithidia luciliae (Bios, Munich, Germany) in 72 sera from SLE patients and 348 control sera contained in our serum bank. Controls included patients with other connective tissue diseases (n=129), rheumatoid arthritis (n=103), inflammatory arthritis (n=51), osteoarthritis (n=32) and healthy subjects (n=33).

A standard curve was constructed employing the positive control serum provided by the manufacturer which by definition contained 100 arbitrary units/ml (U/ml). The cut off value for both assays was determined at 20 U/ml, and this level was not exceeded by sera from HC. At a cut off level of 30 U/ml, anti-histone and anti-chromatin Abs showed moderate sensitivity but quite high specificity for SLE which was close to that of anti-dsDNA Abs. Borderline positive anti-histone Abs (20-30 U/ml) were seen in 14 SLE and 22 control sera. Of note, anti-histone and anti-nucleosome assays from other manufactures were less specific for SLE, presumably due to contaminations with other chromatin proteins (data not shown).
Suppl. Figure 2
Characterization of cellular and humoral autoimmune responses to histone H1 and core histones in human systemic lupus erythematosus

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