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B Cell Proliferation, Somatic Hypermutation, Class Switch Recombination, and Autoantibody Production in Ectopic Lymphoid Tissue in Murine Lupus

Dina C. Nacionales,2* Jason S. Weinstein,2* Xiao-Jie Yan,† Emilia Albesiano,‡ Pui Y. Lee,* Kindra M. Kelly-Scumpia,* Robert Lyons,* Minoru Satoh,* Nicholas Chiorazzi,† and Westley H. Reeves3*

Intraperitoneal exposure of nonautoimmune mice to 2,6,10,14-tetramethylpentadecane (TMPD) causes lupus and the formation of ectopic lymphoid tissue. Although associated with humoral autoimmunity, it is not known whether Ab responses develop within ectopic lymphoid tissue or if B cells only secondarily migrate there. We show that ectopic lymphoid tissue induced by TMPD not only resembles secondary lymphoid tissue morphologically, but it also displays characteristics of germinal center reactions. Proliferating T and B lymphocytes were found within ectopic lymphoid tissue, activation-induced cytidine deaminase was expressed, and class-switched B cells were present. The presence of circular DNA intermediates, a hallmark of active class switch recombination, suggested that class switching occurs within the ectopic lymphoid tissue. Individual collections of ectopic lymphoid tissue (“lipogranulomas”) from the same mouse contained different B cell repertoires, consistent with local germinal center-like reactions. Class-switched anti-RNP autoantibody-producing cells were also found in the lipogranulomas. Somatic hypermutation in the lipogranulomas was T cell-dependent, as was the production of isotype-switched anti-Sm/RNP autoantibodies. Thus, ectopic lymphoid tissue induced by TMPD recapitulates many of the functional characteristics of secondary lymphoid tissue and contains autoantibody-secreting cells, which may escape from normal censoring mechanisms in this location. The Journal of Immunology, 2009, 182: 4226–4236.

Secondary lymphoid tissue, which includes lymph nodes, mucosal-associated lymphoid tissues, and the spleen, is organized to concentrate foreign Ags, placing the cells responsible for mounting an Ag-specific immune response to these Ags (T and B lymphocytes and dendritic cells) in close proximity (1, 2). The tissue is organized into discrete zones containing T cells and dendritic cells (the perioriented lymphoid sheath) and B cells and follicular dendritic cells (the primary follicles). The chemokines CCL19 (ELC) and CCL21 (SLC), which attract T lymphocytes and dendritic cells, and CXCL13 (BLC), which attracts B lymphocytes, play an important role in establishing the compartmentalization of secondary lymphoid tissues into discrete T and B cell zones (3). Ag-specific B cells appear initially at the periphery of the perioriented lymphoid sheath forming primary foci, which are sites of interclonal competition for Ag among unmutated B cells (4). Subsequently, these give rise to a second responding population in the follicle, germinal center B cells. Germinal centers are sites of intraclonal competition for Ag and survival signals between mutated sister lymphocytes (4). The germinal center reaction regulates Ag-specific clonal evolution during the development of B cell memory (5). It is characterized by somatic hypermutation (SHM)6 of Ig complementarity determining regions (CDRs), switch class switch recombination (CSR), clonal expansion (proliferation), and Ag-driven affinity maturation of B cells, expression of activation-induced cytidine deaminase (AID), and a requirement for CD40L.7 T cells (5). B cells in newly formed germinal centers generally are oligoclonal, consisting of one to three clones (6).

The formation of ectopic (tertiary) lymphoid tissue in response to inflammation has been termed “lymphoid neogenesis” because it recapitulates many aspects of secondary lymphoid tissue development (1, 7). Like secondary lymphoid tissue, the organization of ectopic lymphoid tissue is dependent on CCL19 (ELC), CCL21 (SLC), and CXCL13 (BLC) (1). Interestingly, lymphoid neogenesis is strongly associated with autoimmunity and the formation of autoantibodies (8). Autoimmune disorders associated with the formation of ectopic lymphoid tissue include Hashimoto’s thyroiditis, Sjögren’s syndrome, rheumatoid arthritis, and myasthenia gravis (1). We have shown that the i.p. injection of the hydrocarbon pristane (2,6,10,14-tetramethylpentadecane, TMPD) gives rise to the formation of ectopic lymphoid tissue and a chronic immune reaction culminating in the development of lupus (9). In contrast,

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6 Abbreviations used in this paper: SHM, somatic hypermutation; AID, activation-induced cytidine deaminase; CDR, complementarity determining region; CSR, class switch recombination; FDC, follicular dendritic cell; TMPD, 2,6,10,14-tetramethylpentadecane.

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other hydrocarbon oils, such as medicinal mineral oil, induce the formation of ectopic lymphoid tissue but not lupus (10). Inflammatory tissue generated in response to TMPD consists of dendritic cells, monocytes, T cells, and B cells, often organized into discrete zones reminiscent of lymph node architecture, which is vascularized by MECA-79 high endothelial venules (9). The ectopic lymphoid tissue is organized into discrete nodular “lipogranulomas” (11). CCL19, CCL21, and CXCL13 all are expressed in the lipogranulomas and likely play a role in recruiting immune cells into them (9).

In this study we show that the lipogranulomas not only morphologically resemble lymphoid organs but also display some of the characteristics of germinal center reactions, namely proliferation of T and B lymphocytes, T-cell-dependent SHM of Ig-variable regions, expression of AID, and CSR. IgG1 and IgG2a hypergammaglobulinemia induced by TMPD as well as the production of isotype-switched autoantibodies required the presence of T cells. Moreover, autoantibody-secreting cells were present in the lipogranulomas, consistent with the possibility that they can be generated within the ectopic (tertiary) lymphoid tissue.

Materials and Methods

Mice
Four-week-old female BALB/cJ mice were purchased from The Jackson Laboratory and housed in barrier cages. At 3 mo of age, they received a single i.p. injection (0.5 ml) of either TMPD (Sigma-Aldrich) or medicinal mineral oil (Harris Teeter). Peritoneal cells, lipogranulomas, and blood were harvested 12–20 wk later. In some experiments female TCR-deficient (B6.129P2-Tcfrtm1Momm/Tcfrtm1Momm, backcross generation N12) and C57Bl/6J mice (The Jackson Laboratory) were used. These studies were approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry and immunofluorescence

Lipogranulomas were excised from the peritoneal wall after peritoneal lavage, fixed with 4% paraformaldehyde, and embedded in paraffin. Immunohistochemistry was conducted by the Molecular Pathology and Immunology Core at the University of Florida using the Dako Autostainer protocol. Briefly, 4-μm serial sections were deparaffinized and then blocked with Sniper (Biocare Medical). Sections were incubated with rat anti-mouse CD45R (B220) (BD Biosciences), CD3 (Serotec), or Ki-67 (Dako) for 1 h followed by incubation with rabbit anti-rat Ig Abs (Vector Laboratories) for 30 min. Staining was visualized using Mach GT × Rb HRP polymer (Biocare Medical), the chromogen Cardasian diaminobenzidine (Biocare Medical), and Mayer’s hematoxylin counterstain. Tissue sections also were stained with Abs against follicular dendritic cells (FDC-M1; BD Biosciences) and processed for immunohistochemistry as above.

To detect IgM and IgG in the lipogranulomas, deparaffinized sections were stained with either FITC-conjugated goat anti-mouse IgG or IgM (SouthernBiotech), mounted using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories), and examined by fluorescence microscopy.

BrdU labeling of B and T cells

BrdU was administered to BALB/cJ mice (0.2 mg of BrdU i.p. every 4 h for 3 doses) and again 1 day before euthanasia. Peritoneal lipogranulomas from each mouse were excised and pooled. Single-cell suspensions were made by collagenase treatment (9). Spleen cells were also prepared using collagenase and DNAse treatment. The isolated cells were incubated with allophycocyanin BrdU added at pretitrated amounts and incubated for 20 min at 4°C, followed by permeabilization with cold 70% ethanol at −20°C for 3 h. Cells were then analyzed for intracellular staining with anti-Ki-67 Abs (BD Biosciences) using the manufacturer’s protocol. After gating on B220+ or CD4+ lymphocytes, the percentage of Ki-67+ cells was determined by flow cytometry as above.

RT-PCR analysis of AID and class-switched H chain transcripts

Total RNA from individual lipogranulomas excised from TMPD- or mineral oil-treated mice was isolated using TRIzol (Invitrogen) and precipitated with isopropanol. The pellets were washed with cold 75% (v/v) ethanol and resuspended in diethyl pyrocarbonate-treated water. One microgram of RNA was treated with DNase I (Invitrogen) to remove genomic DNA and reverse transcribed to cDNA using SuperScript II strand synthesis system for RT-PCR (Invitrogen). Conventional PCR amplification was conducted in a PTC-100 programmable thermal controller (MJ Research) using primers for AID (forward, GAG GGA GTC AAG AAA GTC ACG CTG GA; reverse, GCC TGA GGT TAG GGT TCC ATC TCA) and β-actin (12). Real-time PCR was performed using SYBR Green core reagents (Applied Biosystems) and a DNA Engine Opticon 2 continuous fluorescence detector (PE). PCR primers were as follows: AID forward, CCT CCT GAC TCG ACT CC; AID reverse, AGG CTG AGG TGA GGC TTC CA; 18S forward, AGG CTA CCA CAT CCA AGG AA; 18S reverse, GCT GGA ATT ACC GCG GCT. AID expression was normalized to the expression of an endogenous control (18S RNA) using the comparative ΔCt method (13). Data are expressed relative to the sample with the lowest expression level. For detecting IgM and IgG1 transcripts, a mixture of eight consensus forward primers (VH1–8) and isotype-specific CμA and Cy1 reverse primers were used (14). Primers were synthesized by Invitrogen. PCR products were analyzed on 1% agarose gels and stained with ethidium bromide.

CSR assay

The occurrence of active CSR in ectopic lymphoid tissue (TMPD or mineral oil-induced lipogranulomas) was evaluated by detecting looped-out circular DNAs as described (15). Briefly, total RNA was isolated from individual lipogranulomas, treated with DNase I, and reverse transcribed to cDNA as above. “Circle transcripts” were amplified as follows: initial denaturation 95°C for 9 min followed by 35 cycles of PCR (94°C for 30 s, 58°C for 60 s) using 0.025 U of TaqDNA polymerase (Invitrogen), 2.0 mM MgCl2, and 1 μM each of isotype-specific I region primers (Iγ1F or Iγ2F) and a CμA reverse primer (15). PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

Ig V(D)J sequence analysis

To determine V(DJ) gene usage, 1 μl of cDNA was amplified using pooled forward (VH1–8) and reverse (VHR2) primers (see Fig. 3A) (14). The reaction was conducted in a 20 μl volume using 1.25 nM pooled VH and 2.5 mM VHR2 primers containing 1× PCR buffer, 1.5 mM MgCl2, 200 μM dNTPs, and 0.05 μM of TaqDNA polymerase (Invitrogen) in a PTC-100 programmable thermal controller (MJ Research) as follows: denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. After 30 cycles, extension was continued at 72°C for an additional 10 min. The PCR product was cloned into a TA vector (pcR4; Invitrogen) and sequenced using an Applied Biosystems model 373 Stretch DNA sequence, 377 DNA sequencer, or 3100 genetic analyzer using a T7 sequencing primer. The determined sequences were verified by sequencing in the reverse direction using a T3 sequencing primer. Vγ, D, and Jγ sequences were identified by searching the Ig-Basic local alignment search tool (BLAST) and ImMunoGeneTics (IMGT)/V-Quest databases using MacVector software (Accelrys).

ELISA

Anti-nRNP/Sn Ag-capture ELISAs were performed as described (16). Ag-coated wells were incubated with 100 μl of mouse sera diluted 1/500 in blocking buffer for 1 h at 22°C, washed three times with TBS/Tween 20, and incubated with 100 μl of alkaline phosphatase-labeled goat anti-mouse IgG or IgM (1/1000 dilution) for 1 h at 22°C. After washing, the plates were developed with p-nitrophenyl phosphate substrate (Sigma-Aldrich). OD at 405 nm (OD405) was read using a VersaMax microplate reader.
FIGURE 1. B and T cell proliferation in lipogranulomas. A, Immunohistochemistry of a TMPD-induced lipogranuloma (serial sections) demonstrating the presence of contiguous B cell (B220+) and T cell (CD3+) zones as well as cellular proliferation, as demonstrated by Ki-67 staining. The bottom right panel shows the absence of cells staining with the FDC marker FDC-M1. B, Flow cytometry of lipogranuloma cells. Gates were set on either the B cell anti-CD45R (B220) or T cells (anti-CD4), and the percentage of cells staining with anti-Ki67 Abs was determined. C, In vivo BrdU labeling of T and B cells in the lipogranulomas and spleens of TMPD-treated mice. Single-cell suspensions were stained with anti-CD45R (B220), anti-CD3, and anti-BrdU Abs. Data are expressed as the percentage of BrdU+B cells or T cells, respectively.

Quantification of plasmablasts
Single-cell suspensions were made using spleen and lipogranuloma tissue from five TMPD-treated BALB/c mice. Cells were stained with allophycocyanin-conjugated anti-B220 and PE-conjugated anti-CD138 Abs (BD Pharmingen) and analyzed by flow cytometry as above.

ELISPOT assay for total Ig
Lipogranulomas and splenocytes from TMPD-treated mice were harvested, analyzed by flow cytometry to determine B cell numbers (anti-CD19), and plated (3 × 10^6 cells/well) in quadruplicate on MultiScreen HTS plates (Millipore) coated with rat IgG anti-mouse L chain Abs (κ- and λ-chain-specific, 3 μg/ml each, from BD Pharmingen). The cells were incubated overnight before adding a combination of alkaline phosphatase-conjugated rat anti-mouse IgG1, IgG2a, IgG3, and IgM were measured by ELISA as described (17).

ELISPOT assay for anti-RNP autoantibodies
The production of anti-U1A (a subset of anti-RNP) autoantibodies in the ectopic lymphoid tissue was also examined by ELISPOT assay. A human full-length U1A cDNA was obtained by RT-PCR (PTC-100 programmable thermal controller (MJ Research)) from normal human PBMC cDNA. The forward primer was GCG GAT CCG CAG TTC CCG AGA CCC GCT CTA ACC AC BanHI and the reverse primer was GCA AGC TTC TAC TTC TTG GCA AAG GAG ATG TTC HindIII. The amplified fragment was inserted between the BanHI and HindIII sites of pET38A (Invitrogen) in-frame with the six-His sequence. The vector was used to transform Escherichia coli BL21 DE3, and recombinant protein was expressed by growing in Luria-Bertani medium containing 10 μg/ml kanamycin and 2 mM isopropyl β-D-thiogalactoside. Four hours later, the bacteria were lysed using 6 M guanidine HCl plus 0.5 mM PMSF and 0.3 trypsin-inhibitory unit (TIU)/ml aprotinin. Recombinant protein was purified using Ni-NTA resin columns (Sigma-Aldrich). The protein was eluted with 6 M urea.

Reactivity with serum anti-RNP autoantibodies from TMPD-treated mice was verified by ELISA. The microtiter plate wells (Immobilizer Amino; Nunc) were coated with 1 μg/ml purified recombinant Ag in BBS overnight at 5°C. The remainder of the ELISA was conducted as described above. Sera from 20 anti-Sm/RNP-positive TMPD-treated mice and 20 untreated controls were tested at a 1/50 dilution followed by 1/1000 alkaline phosphatase-conjugated goat anti-mouse Ig Abs (SouthernBiotech). Using the SoftMax Pro 3.0 software, OD_{405} values were converted to units with a standard curve based on a serially diluted prototype serum.

For the ELISPOT assays, lipogranuloma cells from TMPD-treated BALB/c mice were harvested and plated on MultiScreen HTS plates (Millipore) coated overnight at 4°C with either recombinant U1A protein (5 μg/ml) or BSA followed by alkaline phosphatase-conjugated goat anti-mouse IgG or IgM Abs (1/1000 dilution; SouthernBiotech). Spots were developed overnight with 5-bromo-4-chloro-3-indolyl phosphate/NBT (Pierce Chemical) and counted as above.

Results
Lipogranulomas developing in the peritoneum of TMPD- or mineral oil-treated mice are a form of ectopic lymphoid tissue (9). We investigated whether these structures also exhibit functional characteristics consistent with germinal center reactions, such as SHM, CSR, and Ag-driven, T cell-dependent proliferation of B lymphocytes.

Lymphocyte proliferation in TMPD-induced ectopic lymphoid tissue
As shown previously (9), serial sections of lipogranulomas from TMPD-treated mice revealed contiguous aggregates of B220+ and CD3+ cells (Fig. 1A). Ki-67+ cells were found in the same region,
consistent with the presence of proliferating lymphocytes (Fig. 1A). However, it was difficult to determine from these sections whether T cells, B cells, or both were proliferating. To address this question, pooled lipogranulomas were analyzed by flow cytometry using anti-B220, CD4, and Ki-67 Abs. A small percentage of B220<sup>+</sup> and CD4<sup>+</sup> lymphocytes was Ki-67<sup>+</sup> (Fig. 1B). To confirm the presence of proliferating B and T lymphocytes in the ectopic lymphoid tissue, TMPD-treated mice were injected with BrdU (0.2 mg every 4 h for 3 doses) and euthanized the following day. Incorporation of BrdU by B and T cells in the lipogranulomas and spleen was determined by flow cytometry using anti-BrdU Abs. BrdU<sup>+</sup> B (B220<sup>+</sup>) and T (CD3<sup>+</sup>) cells were present in both the lipogranulomas and the spleen (Fig. 1C). There was a significantly higher percentage of BrdU<sup>+</sup> B and T cells in the lipogranulomas compared with spleen *(p = 0.028)*, indicating that B and T cell proliferation was greater in the ectopic lymphoid tissue than in secondary lymphoid tissue (spleen). Follicular dendritic cells could not be identified in the ectopic lymphoid tissue after staining with FDC-M1 Abs (Fig. 1A), whereas strong staining of follicular dendritic cells could be demonstrated in the spleen (not shown).

**AID expression and CSR in TMPD-induced ectopic lymphoid tissue**

As B cell proliferation in lymphoid follicles is linked to SHM and Ig repertoire diversification (18), we examined the expression of AID, a marker of CSR and SHM, in TMPD and mineral oil lipogranulomas. By RT-PCR, expression of AID was demonstrated in both TMPD and mineral oil induced lipogranulomas but not in peritoneal exudate cells (Fig. 2A). However, the expression appeared lower than in the spleen. Quantitative PCR confirmed that AID expression was lower in lipogranulomas than spleen from TMPD-treated mice, whereas the levels were comparable in lipogranulomas vs spleen of mineral oil-treated mice (Fig. 2A, right). The expression of AID was higher in TMPD or mineral oil lipogranulomas than in peritoneal exudate cells.
Since AID expression is required for Ig class switching, we examined whether IgG-producing B cells were present in the ectopic lymphoid tissue. Class switching to IgG1 and IgG2a, which requires T cells and is characteristic of germinal center reactions, was detected using conventional RT-PCR. Variable levels of \( \mu \) and other CH genes could be detected in nearly all lipogranulomas from either TMPD- or mineral oil-treated mice and high levels were also found in the spleen (Fig. 2B). In contrast, \( \gamma 1 \) H chain mRNA was more abundant in the ectopic lymphoid tissue from TMPD-treated mice in comparison with mineral oil-treated mice. At least low levels of \( \gamma 1 \) H chain were detectable by RT-PCR in 11 out of 12 TMPD lipogranulomas vs 1 out of 12 mineral oil lipogranulomas (Fig. 2B, right).

CSR is accompanied by the looping out of a DNA segment containing \( C_{\mu} \) and other \( C_{\lambda} \) genes generating closed circular
DNAs with isotype-specific I-C/H9262 transcripts. In vitro, these “circle transcripts” are completely removed within 48 h and detection of circle transcripts by PCR is indicative of active CSR (15). We used the presence of circle transcripts to evaluate whether the lipogranulomas were a site of active CSR. Consistent with the data shown in Fig. 2B, circle transcripts were detected in some of the TMPD-induced lipogranulomas (5 out of 31 total), but were rarely detected in mineral oil lipogranulomas (1 out of 17) (Fig. 2C). As a further confirmation, lipogranulomas from mineral oil- or TMPD-treated mice were stained with FITC-conjugated anti-Ig Abs (I-C/H9262- or I-C/H9253-chain-specific) and examined by fluorescence microscopy (Fig. 2D). Mineral oil and TMPD lipogranulomas both contained cells expressing I-C/H9262 H chain, whereas I-C/H9253 H chain was detected only in TMPD lipogranulomas.

Individual lipogranulomas from a single mouse contain different populations of B cells

Germinal center reactions are characterized by oligoclonal expansions of Ag-specific B cells with somatically mutated Ig H and L chains. We therefore examined the B cell repertoire to evaluate whether the lipogranulomas were a site of active CSR. Consistent with the data shown in Fig. 2B, γ2a or γ1 (not shown) circle transcripts were detected in some of the TMPD-induced lipogranulomas (5 out of 31 total), but were rarely detected in mineral oil lipogranulomas (1 out of 17) (Fig. 2C). As a further confirmation, lipogranulomas from mineral oil- or TMPD-treated mice were stained with FITC-conjugated anti-Ig Abs (μ- or γ-chain-specific) and examined by fluorescence microscopy (Fig. 2D). Mineral oil and TMPD lipogranulomas both contained cells expressing μ H chain, whereas γ H chain was detected only in TMPD lipogranulomas.

Table I. Somatic hypermutation of H chains from ectopic lymphoid tissue

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</thead>
<tbody>
<tr>
<td>TMPD</td>
<td>20</td>
<td>2</td>
<td>4</td>
<td>66</td>
<td>39</td>
<td>72</td>
<td>10</td>
<td>1.7</td>
<td>7.2</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>20</td>
<td>2</td>
<td>4</td>
<td>23</td>
<td>11</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

* FR indicates sequences from framework 2 and 3; R, replacement mutation; S, silent mutation; CDR, sequences from CDR 1 and 2.
of the shared sequences in granulomas 139 and 140 (above), the sequences were germline, making it difficult to evaluate whether they were derived from a single clone or two independent clones with the same V(D)J segments.

As suggested by the representative sequences shown in Fig. 4B, the H chain sequences from ectopic lymphoid tissue in mineral oil-treated mice contained fewer somatic mutations than those from TMPD-treated mice. The total somatic mutation frequency in the H chain of mice treated with TMPD was 4.9% (607 mutations/12,466 bases) in contrast to 0.8% (37 mutations/4,336 bases) in mineral oil-treated mice. As shown in Table I, the somatic mutations were found predominantly in the CDR regions of sequences obtained from both TMPD- and mineral oil-induced ectopic lymphoid tissue (replacement/silent mutation ratios of 7.2 and 8, respectively, for the CDR regions of TMPD and mineral oil-treated mice vs 1.7 and 2.0 for the framework regions), suggesting that in both cases, somatic mutations were generated through a process of Ag-selected affinity maturation.

Taken together, these data indicate that the B cells from ectopic lymphoid tissue induced by TMPD or mineral oil in nonimmunized mice were clonally diverse, although there was a suggestion that certain clones may predominate within individual lipogranulomas and that VH36–60, an H chain that is utilized preferentially by B cells with rheumatoid factor or rheumatoid factor-anti-DNA dual reactivity (19, 20), is used considerably more frequently in TMPD- vs mineral oil-induced ectopic lymphoid tissue. We found little evidence for sharing of B cell clones between individual lipogranulomas, as might be expected if the ectopic lymphoid tissue was populated by B cells arising from another location, such as the spleen or lymph nodes.

SHM in TMPD-treated mice is T cell-dependent

SHM of Ig genes occurring during the germinal center reaction usually requires CD40L+ T cells (21). However, both inside and outside of germinal centers, SHM sometimes may be T cell-independent (22–24). To investigate the role of T cells in generating the somatic mutations in H chain sequences from B cells in TMPD-induced ectopic lymphoid tissue, we treated B6.129P2-Tcrbtm1MomTcrdTm1Mom (TCRβ-chain- and δ-chain-deficient) and wild-type C57BL/6J mice with TMPD and analyzed H chain sequences from the lipogranulomas 3 mo later. V(D)J sequences from TCR-deficient mice had a very low rate of SHM (1 mutation/3252 total bases, 0.03%), whereas sequences from C57BL/6J mice had a 20-fold higher rate (12 mutations/1626 bases, 0.7%). Significantly, these mutations were found mainly in the CDR regions (Table II). The greatly increased number of somatic mutations in wild-type vs TCR-deficient mice, clustering of mutations in the CDRs, and the relatively low error rate reported for Taq polymerase (~1 error/10,000 bases) argue that the observed base changes represent true somatic hypermutation and not merely polymerase errors. These data provide further evidence that the SHM seen in

![FIGURE 5](http://www.jimmunol.org/) IgG1 and IgG2a induced hypergammaglobulinemia in TMPD-treated mice is T cell-dependent. A, Serum samples were obtained from wild-type C57BL/6J (WT, n = 5) or B6.129P2-Tcrbtm1MomTcrdTm1Mom (n = 6) mice treated 3 mo earlier with TMPD. IgG1, IgG2a, IgG3, and IgM levels were measured by ELISA and means were compared by the Mann-Whitney U test. B, IgG production in lipogranulomas. Lipogranuloma cells and splenocytes from two mice were tested in quadruplicate for T cell-dependent Ig secretion (IgG1 + IgG2a + IgG2b) by ELISPOT assay. C, Quantification of plasmablasts in spleen and lipogranulomas. Pooled lipogranuloma and spleen cells from four TMPD-treated BALB/cJ mice were stained with anti-B220 and anti-CD138 Abs and analyzed by flow cytometry.

### Table II. Somatic hypermutation in ectopic lymphoid tissue from TCR-deficient mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. Lipogranulomas</th>
<th>No. Sequences</th>
<th>Framework R (%)</th>
<th>Framework S (%)</th>
<th>CDRs R (%)</th>
<th>CDRs S (%)</th>
<th>R/S ratio</th>
</tr>
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| WT        | 2                   | 6             | 0.08            | 0.32            | 1.30       | 0.50       | 0.25      | 2.5
| TcR KO    | 4                   | 12            | 0               | 0               | 0.13       | 0          | 0         | 2.0

* VM indicates wild type; R, replacement mutations; S, silent mutations; FR, framework regions; CDR, complementarity determining regions.

* Unable to calculate (0 denominator).
ectopic lymphoid tissue from TMPD-treated mice was generated through a germinal center-like reaction.

**TMPD-induced hypergammaglobulinemia and autoantibody production are also T cell-dependent**

Two of the characteristic immune abnormalities induced by TMPD treatment are induction of polyclonal hypergammaglobulinemia and the development of IgM and IgG autoantibodies, such as anti-RNP/Sm antibodies associated with SLE. Since CSR to γ1 and γ2a H chain occurs in TMPD-induced ectopic lymphoid tissue (Fig. 2C), we investigated whether the increased production of polyclonal serum IgG and IgG autoantibodies requires the presence of T cells. Total Ig levels were determined (ELISA) in sera from TCR-deficient and wild-type mice treated with TMPD 3 mo earlier. Levels of IgM and IgG3 were comparable in wild-type vs knockout mice (Fig. 5A), consistent with the fact that IgM and IgG3 Ab production is largely T cell-independent. However, IgG1 and IgG2a levels were significantly higher in the wild-type mice (Mann-Whitney U test, p = 0.008 and p = 0.03, respectively), indicating that the TMPD-induced polyclonal increase in these isotypes was T cell mediated.

To determine whether some of the T cell-dependent Ig production was derived from B cells present in the lipogranulomas, ELISPOT assays were performed using cells isolated from collagenase-treated ectopic lymphoid tissue from an anti-RNP-positive TMPD-treated mouse or an anti-RNP-negative mouse treated with medicinal mineral oil. Representative of three experiments, IgG ELISPOT assay with purified U1A or BSA Ags, using cells isolated from lipogranulomas (Lipogran) or spleens of anti-U1A-positive mice (n = 5). The frequencies of Ag-specific spots are expressed per 50,000 B cells. The frequency of anti-U1A spots was higher in lipogranulomas than in spleen (p = 0.01, Mann-Whitney U test) and the frequency of anti-U1A spots was higher than the frequency of anti-BSA spots (p = 0.03, Mann-Whitney U test).
the two locations secreted comparable amounts of polyclonal Ig. We previously showed that after immunization with exogenous Ag, T cells from the lipogranulomas secrete IL-21, which has been shown to play a role in plasma cell differentiation (25). These data indicate that lipogranuloma cells actively secrete Abs of T cell-dependent isotypes.

Finally, we examined the role of ectopic lymphoid tissue induced by TMPD in the pathogenesis of lupus-associated autoantibodies against the U1 small ribonucleoprotein (anti-Sm and anti-RNP antibodies). IgM anti-RNP/Sm autoantibodies (ELISA) were detected at low, but comparable, levels in the sera of wild-type and TCR knockout mice (Fig. 6A). In contrast, IgG anti-nRNP/Sm autoantibodies were produced by wild-type animals, but the levels in TCR-deficient mice were not statistically different than those in untreated controls (Fig. 6B). These experiments suggested that not only was the induction of polyclonal IgG1 and IgG2a by TMPD T cell-dependent (Fig. 5), but also the appearance of class-switched serum autoantibodies required T cells, a characteristic of autoantibodies generated during germinal center reactions.

The presence in ectopic lymphoid tissue of B cells producing class-switched autoantibodies against the U1 small ribonucleoprotein was investigated using an ELISPOT for anti-U1A (anti-RNP) autoantibodies. The purified recombinant U1A Ag used for ELISPOT assays was reactive with sera from 19 out of 20 anti-RNP and/or anti-Sm-positive TMPD-treated BALB/c mice, but not with 20 normal mouse sera (Fig. 6C). As shown in Fig. 6D, left panel, large numbers of IgM anti-U1A autoantibody-secreting cells were detected in cells obtained from TMPD-induced ectopic lymphoid tissue, but not in ectopic lymphoid tissue induced by medicinal mineral oil, which does not induce serum anti-RNP or anti-Sm autoantibodies. Similarly, IgG anti-U1A autoantibody-secreting cells were detected in TMPD-induced ectopic lymphoid tissue, but not in mineral oil-induced ectopic lymphoid tissue (Fig. 6D, right panel). We next compared the frequencies of anti-U1A-secreting B cells in the lipogranulomas vs spleen of mice that were positive for serum anti-RNP autoantibodies (Fig. 6E). A substantial difference in the frequency of anti-U1A-secreting B cells in the lipogranulomas vs the spleen was observed (p = 0.01, Mann-Whitney U test). There also was a significant difference in the frequencies of cells in the lipogranulomas secreting anti-U1A autoantibodies vs Abs against a control foreign Ag, BSA (p = 0.03), suggesting that autoantibody-producing cells may preferentially localize to or develop within the ectopic lymphoid tissue.

These experiments indicate that class-switched autoantibody-producing cells were present within the ectopic lymphoid tissue and were secreting autoantibodies. Taken together, the data in Figs. 5 and 6 suggest that the increased polyclonal IgG as well as the IgG anti-RNP autoantibodies in the sera of TMPD-treated mice are likely to be at least partially derived from B cells/plasma cells in the ectopic lymphoid tissue.

Discussion
Structures morphologically and developmentally resembling secondary lymphoid organs (ectopic lymphoid tissue) form at the sites of chronic inflammation, a process known as lymphoid neogenesis (1, 7, 26). There is a strong association of lymphoid neogenesis with humoral autoimmunity (1). However, the role of ectopic lymphoid tissue in initiating immune/autoimmune responses, as opposed to serving as a reservoir for B lymphocytes previously activated elsewhere, has not been fully defined. In New Zealand Black/New Zealand White lupus mice, plasma cells are activated in the spleen and secondarily migrate to inflamed tissues, such as the kidney (27, 28), whereas in patients with rheumatoid arthritis or Sjögren’s syndrome ectopic lymphoid tissue may represent a site of Ag-dependent B cell differentiation consistent with a true germinal center reaction (29–31).

Intrapерitoneal exposure to TMPD induces lupus in mice (32, 33) with formation of ectopic lymphoid tissue (9), consisting of lipogranulomas, discrete nodules attached to the mesothelial lining of the peritoneal cavity (11). In certain strains of mice, notably BALB/cAnPt, plasma cell neoplasms develop in the lipogranulomas after several months (34). Closer examination shows that the lipogranulomas morphologically resemble secondary lymphoid tissue, with discrete B cell and T cell-dendritic cell rich zones, MECA-79+ high endothelial venules, and the expression of an array of lymphoid chemokines characteristic of developing lymphoid tissue (9). Following immunization, T cells and B cells specific for exogenous test Ads (4-hydroxy-3-nitrophenyl acetyl-keyhole limpet hemocyanin (NP-KLH) and NP-OVA) are enriched in TMPD lipogranulomas and individual lipogranulomas frequently contain monoclonal populations of proliferating NP-specific B cells along with proliferating carrier-specific T cells (25).

The objective of the present study was to see if autoimmune responses can develop within foci of chronic inflammation in lupus. T and B cell proliferation and AID expression as well as SHM and CSR of Ig genes were found in TMPD-induced lipogranulomas. Additionally, we report that B cells actively secreting a prototypical lupus autoantibody, anti-U1A, are enriched in the ectopic lymphoid tissue (Fig. 6E). A key question is whether the local production of these autoantibodies is stimulated by cognate T-B interactions within the ectopic lymphoid tissue (consistent with a germinal center reaction) or by Ag-independent mechanisms, such as TLR signaling. Although unlike germinal centers, TMPD-induced ectopic lymphoid tissue did not contain FDC-M1+ FDCs (Fig. 1A), FDC-M1+ FDCs have been described (35).

In humans, FDCs have been reported in lymphoid neogenesis arising in the stomach, rheumatoid synovium, salivary glands, and other locations (36–38), raising the possibility that the germinal center-like structures found in these sites are sites of cognate T-B interaction involved in autoantibody production. Conversely, autoantibodies can be produced extrafollicularly by B cells located at the border between the T cell zone and the red pulp of the spleen (23, 39, 40). This is a site where T cell-independent responses to foreign Ads occur, and it has been shown that in AM14 rheumatoid factor transgenic mice, the activation of autoantibody production requires TLR signaling but not T cells (41).

The presence of AID and circle intermediates in TMPD-induced ectopic lymphoid tissue (Fig. 2) strongly suggests that B cell activation occurs locally. AID, an enzyme required for CSR and SHM (42, 43), is expressed in germinal centers (44). Additionally, the presence of circle transcripts (Fig. 2C), transient intermediates of CSR, which at least in vitro disappear within 48 h of being generated (15), strongly suggests that the ectopic lymphoid tissue is a site of CSR, arguing against the possibility that isotype-switched B cells secondarily migrate there. However, although the presence of circle transcripts is suggestive of local CSR, we cannot at present exclude the possibility that circle transcripts are degraded more slowly under in vivo conditions.

Additionally, although characteristic of germinal center reactions, AID expression and CSR can be induced in B cells by TLR signaling (45–47). Thus, even though B cell activation occurs locally, since the U1 small ribonucleoprotein carries an endogenous TLR7 ligand (48), we cannot completely exclude the possibility that anti-Sm/RNP autoantibody production in the ectopic lymphoid tissue is Ag-independent and driven by TLR7 signaling, as has been reported for other autoantibodies. For instance, when injected with an IgG2a anti-chromatin Ab, AM14 transgenic mice deficient in T cell receptors generate AM14 (rheumatoid factor)
Disclosures

The authors have no financial conflicts of interest.

References


