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Colocalization of Antigen-Specific B and T Cells within Ectopic Lymphoid Tissue following Immunization with Exogenous Antigen

Jason S. Weinstein,* Dina C. Nacionales,* Pui Y. Lee,* Kindra M. Kelly-Scumpia,* Xiao-Jie Yan,§ Philip O. Scumpia,† Dustin S. Vale-Cruz,‡ Eric Sobel,* Minoru Satoh,* Nicholas Chiorazzi,§ and Westley H. Reeves²*

Chronic inflammation promotes the formation of ectopic lymphoid tissue morphologically resembling secondary lymphoid tissues, though it is unclear whether this is a location where Ag-specific immune responses develop or merely a site of lymphocyte accumulation. Ectopic lymphoid tissue formation is associated with many humoral autoimmune diseases, including lupus induced by tetramethylpentadecane in mice. We examined whether an immune response to 4-hydroxy-3-nitrophenyl acetyl-keyhole limpet hemocyanin (NP-KLH) and NP-OVA develops within ectopic lymphoid tissue (“lipogranulomas”) induced by tetramethylpentadecane in C57BL/6 mice. Following primary immunization, NP-specific B cells bearing V186.2 and related heavy chains as well as λ-light chains accumulated within ectopic lymphoid tissue. The number of anti-NP-secreting B cells in the ectopic lymphoid tissue was greatly enhanced by immunization with NP-KLH. Remarkably, the H chain sequences isolated from individual lipogranulomas from these mice were diverse before immunization, whereas individual lipogranulomas from single immunized mice had unique oligo- or monoclonal populations of presumptive NP-specific B cells. H chain CDR sequences bore numerous replacement mutations, consistent with an Ag-driven and T cell-mediated response. In mice adoptively transferred with OT-II or DO11 T cells, there was a striking accumulation of OVA-specific T cells in lipogranulomas after s.c. immunization with NP-OVA. The selective colocalization of proliferating, Ag-specific T and B lymphocytes in lipogranulomas from tetramethylpentadecane-treated mice undergoing primary immunization implicates ectopic lymphoid tissue as a site where Ag-specific humoral immune responses can develop. This has implications for understanding the strong association of humoral autoimmunity with lymphoid neogenesis, which may be associated with deficient censoring of autoreactive cells. The Journal of Immunology, 2008, 181: 3259–3267.

Lymphoid neogenesis, the formation of ectopic (tertiary) lymphoid tissue in response to inflammation (1, 2), is associated with the production of autoantibodies in several diseases including Sjögren’s syndrome, rheumatoid arthritis, and myasthenia gravis (3). Whether ectopic lymphoid tissue participates directly in generating autoreactive B cells (e.g., by allowing the autoreactive cells to escape self-tolerance) or indirectly as a site where mature Ab-secreting cells can persist (4) is unknown. Intraperitoneal injection of non-autoimmune-prone mice such as BALB/c with the hydrocarbon oil 2,6,10,14-tetramethylpentadecane (TMPD)⁵ triggers the formation of ectopic lymphoid tissue (“lipogranulomas”) and the development of lupus-like autoimmune disease with autoantibodies against small nuclear ribonucleoproteins and dsDNA, immune complex-mediated glomerulonephritis, arthritis, and vasculitis (5–8). Within the ectopic lymphoid tissue induced by TMPD are CD11c⁺ dendritic cells (DCs) expressing the costimulatory molecule CD86. Expression of the lymphoid chemokines CXCL13 (BLC), CCL19 (ELC), and CCL21 (SLC) is likely to play a role in the accumulation of B cells, T cells, and DCs in TMPD-induced tertiary lymphoid tissue (⁹). Although individual TMPD-induced lipogranulomas bear some resemblance histologically to germinal centers, there are differences, including the absence of peanut agglutinin⁺ B cells and FDC-MI⁺ follicular DCs (⁷). Nevertheless, proliferating (Ki-67⁺) lymphocytes can be demonstrated in these structures. Moreover, the B cells bear somatically mutated and isotype-switched Ig heavy chains (D. C. Nacionales, J. S. Weinstein, X. J. Yan, E. Albesiano, P. Y. Lee, K. M. Kelly-Scumpia, R. Lyons, M. Satoh, N. Chiorazzi, and W. H. Reeves, submitted), suggesting that lipogranulomas may support Ag-specific immune responses and may be a location where tolerance to autoantigens can be overcome.

The germinal center reaction regulates Ag-specific clonal evolution during the development of B cell memory (10). B cells in newly formed germinal centers may be oligoclonal (11), whereas those in TMPD-induced lipogranulomas are usually more diverse (D. C. Nacionales et al., submitted). The existence in TMPD-treated mice of occasional lipogranulomas containing oligoclonal B cells is currently unexplained.

*Division of Rheumatology and Clinical Immunology and Center for Autoimmune Disease, ‡Department of Surgery, and §Department of Anatomy and Cell Biology, University of Florida, Gainesville, FL 32610; and Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health System, Manhasset, NY 11030

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2 Address correspondence and reprint requests to Dr. Westley H. Reeves, Division of Rheumatology and Clinical Immunology, University of Florida, PO Box 100221, Gainesville, FL 32610. E-mail address: whreeves@ufl.edu

3 Abbreviations used in this paper: TMPD, tetramethylpentadecane; DC, dendritic cell; NP-KLH, 4-hydroxy-3-nitrophenyl acetyl-keyhole limpet hemocyanin.

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Previous studies have shown that following immunization with 4-hydroxy-3-nitrophenyl acetyl-keyhole limpet hemocyanin (NP-KLH), two anatomically and phenotypically distinct populations of Ab-forming cells arise in the spleen. As early as 2 days after immunization, primary foci consisting of Ag-binding B cells are seen along the periphery of the periarteriolar lymphoid sheaths (12). Initially these foci expand, but by day 14, they disappear, giving rise to a second responding population in the follicle, germinal center B cells, which appear on day 8–10 and persist at least until day 30 postimmunization. The primary foci are sites of interclonal competition for Ag among unmutated B cells, whereas germinal centers are sites of intraclonal competition between mutated sister lymphocytes (12), as well as interclonal competition between pre-existing germinal center B cells and follicular “visitors” that can join the germinal center reaction if they have a sufficiently high Ag-binding affinity (13).

In the present study, we analyzed Ag-specific B and T cells in TMPD-induced ectopic lymphoid tissue at 12 days after primary s.c. immunization with a test Ag, NP-KLH. We show that oligoclonal populations of NP-specific B cells develop in TMPD-induced ectopic lymphoid tissue and may displace or overgrow more diverse populations of non-NP specific B cells present before immunization. Along with the hapten-specific B cells, the TMPD-induced lipogranulomas contain carrier-specific T cells, strongly suggesting that ectopic lymphoid tissue can participate directly in the generation of Ag-specific B cell responses.

Materials and Methods

Mice

Six-week-old female C57BL/6, BALB/cJ, T cell transgenic C.Cg-Ttg(D011.10)1Dlo/J (D011.10), and C57BL/6-Tg(TcrαTcrβ)425Cbn/J (OT-II) mice were purchased from The Jackson Laboratory and housed in barrier cages. At 2 mo of age, C57BL/6 and D011.10 mice received 0.5 ml of 2, 6, 10, 14 TMPD (Sigma-Aldrich) i.p. or were left untreated. The mice were injected 3 mo later s.c. in the lower abdomen with 100 μg of NP<sub>17–19</sub>-conjugated KLH (NP-KLH; Biosearch Technologies) precipitated in alum (Pierce). Lipogranulomas, spleen, and blood were harvested 4 to 12 days later. These studies were approved by the Institutional Animal Care and Use Committee.

Anti-NP IgM and IgG ELISA

Preeimmune sera and sera obtained at the time of euthanasia were tested for IgM and IgG anti-NP Abs (ELISA). Microtiter plates were coated with NP<sub>15–24</sub> or NP<sub>21–30</sub>–conjugated BSA (Biosearch Technologies). Serially diluted serum samples were added for 1 h at room temperature. Anti-NP IgM and IgG Abs were detected using alkaline phosphatase conjugated goat anti-mouse IgM or IgG Abs (1/1000 dilution; BD Biosciences) followed by phosphate substrate (Sigma-Aldrich). OD was converted to concentration based on standard curves with sera from C57BL/6 mice immunized with NP-KLH using a four-parameter logistic equation (Softmax Pro 3.1 software; Molecular Devices).

BrdU labeling of B and T cells

BrdU was administered to BALB/c mice (0.2 mg BrdU i.p. every 4 h for 3 doses) and again one day before euthanasia. Single-cell suspensions of lipogranulomas or spleen tissue were made by collagenase treatment (7). Single-cell suspensions of lipogranulomas or spleen tissue were made by collagease treatment (7). Lipogranulomas and spleen were fixed and embedded as previously described (7). Sections (4 μm) were stained with HRP-conjugated goat anti-IgM Abs (Southern Biotechnology Associates), developed with diaminobenzidine (Vector Laboratories), and viewed under a light microscope.

Anti-NP ELISPOT assay

Lipogranulomas and spleen (10<sup>4</sup> cells/well) from NP-KLH/TMPD treated mice were collagease treated as described (7) and single-cell suspensions were plated on Multiscreen HTS plates (Millipore) coated with NP<sub>21–30</sub>-BSA. Lipogranulomas and spleen cells from non-immunized TMPD treated mice were used as the negative control. The cells were incubated overnight before adding alkaline phosphatase-conjugated goat anti-IgM Abs. Spots were developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT (Pierce) and incubated overnight before counting using a dissecting microscope.

V<sub>H</sub> gene sequences

Lipogranulomas and spleen were harvested at day 12 and mRNA was isolated using TRIZol reagent (Invitrogen). The pellets were washed with cold 75% (v/v) ethanol and resuspended in diethyl pyrocarbonate-treated water. One μg of RNA was treated with DNase I (Invitrogen) to remove genomic DNA and reverse transcribed to cDNA using Superscript First Strand Synthesis System for RT-PCR (Invitrogen). PCR amplification of Ig H chain cDNA was performed using a mixture of 8 forward primers (VH1–8) and a consensus reverse primer (VHR2) as described (14) (D. C. Nacionales et al., submitted). The PCR products were cloned into TOPO vector (pCR4; Invitrogen), and the VDH chain sequences were determined by dye-dissolving and analyzed using the Macvector V 7.2.3 program (Accelrys Inc.).

Transfer of Ag-specific T cells

A total of 5 × 10<sup>5</sup> CD4<sup>+</sup> T cells from OT-II or D011.10 mice were transferred i.v. to C57BL/6 or BALB/cJ TMPD treated recipients. The mice were immunized s.c. 3 days after T cell transfer with 200 μg NP<sub>17–19</sub>-OVA precipitated in alum. Single-cell suspensions of the draining lymph nodes, spleen, and lipogranulomas were prepared as above 7 days after immunization. Lipogranuloma cell suspensions from C57BL/6J recipients of OT-II T cells were analyzed by FACS using anti-CD3-FITC, anti-CD4-APC, anti-Vo2-PE, and anti-Vo5-Biotin-Av/Pac Blue Abs (BD Biosciences). BALB/cJ recipients of D011.10 T cells were analyzed using anti-D011.10 (KJ-26)-APC Abs (Invitrogen/Caltag).

T cell proliferation assay

DO11.10 mice were immunized 3 mo after TMPD or saline treatment with 50 μg of specific peptide corresponding to amino acids 323–339 of OVA (OVA<sub>323–339</sub>; Genscript Corporation) in alum. CD4<sup>+</sup> T cells were sorted 5 days later using MACS anti-CD4 beads (Miltenyi Biotec). CD4<sup>+</sup> T cells totaling 2.5 × 10<sup>5</sup> were cultured with irradiated (3000 B), CD4<sup>+</sup>–depleted APCs (2.5 × 10<sup>5</sup> cells/well) in quadruplicate. A total of 2.5 μg/ml soluble anti-CD3 or 10 μg/ml of OVA<sub>323–339</sub> was added for 48–72 h in a total volume of 200 μl of complete RPMI medium. One μCi [1<sup>H</sup>]thymidine (Amershams Biosciences) was added for the final 16 h of culture, and proliferation was determined using a liquid scintillation counter.

PCR analysis of T cell cytokines

Lipogranulomas and spleen were harvested 10 days after immunization with NP-KLH followed by isolation of mRNA and cDNA synthesis as described above. PCR amplification of β-actin, IL-4, IFN-γ, and IL-21 was performed using the following primers: β-actin forward: (TGGATCACTTGG GCATCCATGAAAAC); β-actin reverse (TAAAACGGCAGCTGAATTACA GTCCG); (IL-4 forward: (CGAAGAACACCACAGAGAGTGAGCT); IL-4 reverse: (GTCACAGTTTTC GCTGACTGAACTCAGATTGTAG); IFN-γ forward: (CGAAGAACACCACAGAGAGTGAGCT); IFN-γ reverse: (GTCACAGTTTTC GCTGACTGAACTCAGATTGTAG); IL-21 forward: (ATGAGAGGACCTCGTTGCTG); IL-21 reverse: (GTGCTGGTTGCGTCTGTA). One μl of cDNA was added to a mixture containing 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 400 μM dNTPs, 0.025 U of TaqDNA polymerase (Invitrogen), 1 μM each of forward and reverse primers, and diethyl pyrocarbonate-water in a 20 μl volume. Amplification was conducted for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 30 s, extension at 72°C for 45 s, and a final extension of 72°C for 10 min in a PTC-100 Programmable Thermal Controller (MJ Research). PCR products were visualized on 1% agarose gel.
Results

Ag-specific B cell responses in ectopic lymphoid tissue

Intraperitoneal injection of TMPD leads to the formation of ectopic lymphoid tissue containing B and T lymphocytes as well as activated DCs (7). We asked whether lipogranulomas are a site where specific T cell-dependent Ab responses develop. TMPD-treated B6 mice were immunized s.c. with NP-KLH. A, Affinity of anti-NP Abs developing in TMPD-treated mice vs controls (no Rx) immunized with NP-KLH. Serum binding activity (measured in arbitrary units using a standard curve) was measured using NP30-BSA (low and high avidity/affinity Abs) and NP3-BSA (high avidity/affinity Abs). B, In vivo BrdU labeling of T and B cells in the lipogranulomas and spleens of TMPD-treated and NP-KLH immunized mice (n = 3). Single-cell suspensions were stained with anti-CD45R (B220) and anti-CD3 Abs and with an anti-BrdU Ab. Data are expressed as the percentage of BrdU+ B cells or T cells, respectively, at 4, 8, 10, or 14 days after immunization with NP-KLH.

FIGURE 1. Serum anti-NP response after immunization with NP-KLH. B6 mice injected with TMPD 3 mo earlier were immunized with NP-KLH. At day 12, sera were tested for IgM (A) and IgG (B) anti-NP Abs by ELISA using NP-BSA. There was a significant increase in both IgM and IgG anti-NP from preimmune sera to day 12 immune sera. C, Isotypes of anti-NP Abs (ELISA) from mice either pretreated or not pretreated with TMPD before immunization with NP-KLH. D, Affinity of anti-NP Abs developing in TMPD-treated mice vs controls (no Rx) immunized with NP-KLH. Serum binding activity (measured in arbitrary units using a standard curve) was measured using NP30-BSA (low and high avidity/affinity Abs) and NP3-BSA (high avidity/affinity Abs). E, In vivo BrdU labeling of T and B cells in the lipogranulomas and spleens of TMPD-treated and NP-KLH immunized mice (n = 3). Single-cell suspensions were stained with anti-CD45R (B220) and anti-CD3 Abs and with an anti-BrdU Ab. Data are expressed as the percentage of BrdU+ B cells or T cells, respectively, at 4, 8, 10, or 14 days after immunization with NP-KLH.
stimulated lymphocyte proliferation within ectopic lymphoid tissue and that the magnitude of this proliferative response was comparable to that in a secondary lymphoid organ, the spleen.

**OVA-specific T cells localize and expand in ectopic lymphoid tissue**

To further address whether ectopic tissue facilitates the development of de novo immune responses, we analyzed Ag-specific CD4+ T cell responses within the lipogranulomas following immunization. CD4+ OVA peptide-specific T cells were transferred from either OT-II or DO11.10 mice into TMPD treated recipients. Either PBS or CD4+ OVA-specific T cells from OT-II mice were injected into TMPD-treated B6 mice, followed by immunization with NP-OVA 3 days later. We identified the OVA transgenic T cells (Vα2′Vβ5′CD4+ cells) 7 days after immunization (16) in various lymphoid tissues using flow cytometry. There was a significant increase of Vα2′Vβ5′CD4+ T cells in the draining lymph nodes and lipogranulomas from mice injected...
with OT-II T cells compared with the control PBS injection (Fig. 2A). As expected, the DO11.10 Ag-specific T cells also were present at increased frequency in the lipogranulomas of BALB/c mice after immunization (Fig. 2B). Transfer of DO11.10 T cells to non-immunized mice verified that the increased numbers of Ag-specific T cells in the lipogranulomas were not merely related to the transfer of CD4+ T cells (Fig. 2B). We also treated DO11.10 mice with TMPD and 3 mo later immunized with OVA323–339. The lipogranulomas contained almost exclusively Ag-specific KJI-26 CD4+ T cells 7 days after immunization, whereas both spleen and draining lymph nodes contained a population of KJI-26+ CD4+ T cells (Fig. 2C). In contrast, lipogranulomas from non-immunized mice contained a population of non-transgenic (KJI-26-) T cells. We further assessed the presence of OVA-specific T cells in the lipogranulomas by isolating CD4+ T cells from TMPD-treated DO11.10 mice and stimulating in vitro with OVA323–339. Ag-specific CD4+ T cells from lipogranulomas, spleen, and draining lymph nodes all proliferated similarly (Fig. 2D). The expansion of Ag-specific CD4+ T cells in the lipogranulomas upon immunization coupled with their ability to proliferate when stimulated in vitro provides evidence that ectopic lymphoid tissue is a site of Ag-specific T cell activation and proliferation.

To further demonstrate that the T cells in the lipogranuloma are active participants in an Ag-specific immune response, we looked at the production of T cell inflammatory cytokines in lipogranulomas and spleen after immunization with the test Ag NP-KLH. Compared with the spleen, lipogranulomas from the same mouse expressed higher levels of IFN-γ mRNA but lower levels of IL-4 and IL-21 (Fig. 2E). Thus, following immunization, T cells in the lipogranulomas were proliferating (Fig. 1E), there was an increased number of Ag-specific CD4+ cells (Fig. 2, B and C), and there was production of T cell cytokines, particularly IFN-γ (Fig. 2E), consistent with the presence of effector T cells.

**NP-specific B cells and anti-NP Ab production in ectopic lymphoid tissue**

In view of the preferential pairing of λ1 l-chains with V186.2 H chains, paraffin-embedded tissue was stained for κ and λ light chains (Fig. 3A). Lipogranulomas from both immunized and non-immunized mice contained large numbers of κ L chain bearing B cells (Fig. 3B). In contrast, lipogranulomas from a TMPD-treated mouse immunized with NP-KLH were +50% and spleen (+50%) from the same mouse. All of the sequences recovered from lipogranulomas bore V186.2, CH10, V303, V102, or V124 vs 62.6% of the sequences recovered from the spleen (representative of three independent experiments).
cells, showing that immunization does not substantially affect the total number of B cells in the lipogranulomas. The lipogranulomas from mice immunized with NP-KLH (day 12) and two preimmune (not immunized) mice. V-D-J sequences were amplified from cDNA by PCR and sequenced to determine V_{H}, D, and J_{H} usage. Boxed sequences use V_{H} sequences associated with anti-NP reactivity. Related sequences are shown in the same format. A, H chain sequences are shown from spleen and lipogranulomas of two mice immunized with NP-KLH (day 12) and two preimmune (not immunized) mice. V-D-J sequences were amplified from cDNA by PCR and sequenced to determine V_{H}, D, and J_{H} usage. Related sequences are shown in the same format. B, Graphical representation of the percentage of anti-NP H chains in tissues from immunized or unimmunized mice.

Table I. V186.2 sequences from mice undergoing primary NP-KLH immunization

<table>
<thead>
<tr>
<th>Timepoints</th>
<th>Total No. of Sequences</th>
<th>No. of Mutated V186.2</th>
<th>No. of FR S Mutations (V186.2)</th>
<th>No. of FR R Mutations (V186.2)</th>
<th>No. of CDR S Mutations (V186.2)</th>
<th>No. of CDR R Mutations (V186.2)</th>
<th>Ratio of Replacement (R) to Silent (S) Mutations in V186.2 sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipogranuloma</td>
<td>31</td>
<td>9</td>
<td>8</td>
<td>0</td>
<td>11</td>
<td>8</td>
<td>0.72</td>
</tr>
<tr>
<td>Spleen</td>
<td>31</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>13</td>
<td>17</td>
<td>1.3</td>
</tr>
</tbody>
</table>
FIGURE 6. CDR1 and CDR2 sequences from H chains isolated from lipogranulomas. Sequences of H chains from mouse 1, lipogranuloma 2 (V303-DSP2.9-JH1) and mouse 2, lipogranuloma 1 (V303-DSP2.8-JH4) are aligned. Somatic mutations are indicated. Replacement mutations capitalized, silent mutations lower case.

Discussion

There are many examples of structures resembling secondary lymphoid tissue arising at sites of chronic inflammation (2), but it remains unclear whether this ectopic lymphoid tissue is a site of cognate T-B interactions. Specifically, although autoantigen-specific B cells have been reported in ectopic lymphoid tissue (19, 20), it is not known whether immune responses actually develop there or whether Ag-specific B cells arising in secondary lymphoid tissues subsequently colonize the ectopic lymphoid tissue. Here, we addressed this question by active s.c. immunization coupled with tracking of Ag-specific B and T lymphocytes to peripheral ectopic lymphoid tissue induced by TMPD. To our knowledge, this is the first study to show that both hapten-specific B cells and proliferating, carrier-specific T effector cells are present within ectopic lymphoid tissue.

Our data strongly suggest that cognate Ag-specific T-B interactions occur in ectopic lymphoid tissue. Following s.c. immunization with Ags such as NP-OVA, T and B cell proliferation was seen in the TMPD-induced ectopic lymphoid tissue (Fig. 1E) and both OVA-specific T cells and NP hapten-specific B cells
accumulated there (Figs. 2 and 3). T cells in the ectopic lymphoid tissue also produced IFN-γ and other cytokines (Fig. 2E). H chain sequences isolated from ectopic lymphoid tissue were highly enriched for V186.2 and other H chains known to generate NP-specific Abs (Figs. 4 and 5), and the proportion of such sequences was higher than in the spleen. Lipogranulomas also exhibited strong λ L chain staining consistent with an anti-NP response (Fig. 3). Moreover, anti-NP Ab secreting cells were enriched in ectopic lymphoid tissue in comparison with the spleen. Taken together, these data suggest that Ag-specific B cell and T cell responses may preferentially develop within the ectopic lymphoid tissue.

Individual lipogranulomas from preimmune mice contain relatively diverse populations of B cells (Fig. 5; D. C. Nacionales et al., submitted). In contrast, following s.c. immunization, the B cells present in individual lipogranulomas were highly oligo-clonal or even monoclonal (Figs. 5 and 6) and preferentially used H chains previously reported in anti-NP responses. Oligo-clonal B cell expansions also are seen in individual germinal centers microdissected from secondary lymphoid tissues (11), suggesting that individual lipogranulomas from TMPD-treated mice are in some respects analogous to single germinal centers. The oligo-clonal B cell proliferation apparent in TMPD-induced ectopic lymphoid tissue is consistent with previous observations in ectopic lymphoid tissues from rheumatoid arthritis, Sjögren’s syndrome, and myasthenia gravis patients (19, 21, 22).

A key question is whether the B cells in ectopic lymphoid tissue develop in situ or migrate into the ectopic lymphoid tissue from secondary lymphoid organs, such as the lymph nodes or spleen. In view of the timing of immunization and the V_{H}I1 sequences obtained, it is unlikely that the B cells found in lipogranulomas 10–12 days after immunization originated from the germinal centers of secondary lymphoid tissues followed by migration to the tertiary lymphoid tissues. The fact that individual lipogranulomas contained non-overlapping and unrelated sets of clonal B cells also suggests they were not “seeded” with the products of previous germinal center reactions in the lymph nodes or spleen. Moreover, the sequences recovered from spleen did not overlap with the lipogranuloma sequences.

We did not find the extensive clonal trees reported previously from spleen of MRL mice (23). However, extensive clonal trees were not seen in individual germinal centers, either (11), and the sequences illustrated in Fig. 6 (mouse 2, granuloma 1) are not that dissimilar from those reported previously from individual germinal centers. The lack of clonal trees could reflect the relatively small number of sequences analyzed per lipogranuloma or a lower rate of somatic hypermutation in ectopic lymphoid tissue vs secondary lymphoid tissues.

There are other important differences between the ectopic lymphoid tissue induced by TMPD and authentic germinal center reactions, notably the absence of well-developed follicular DC networks and PNA^- B cells (7). ELISPOTs were larger using spleen cells vs cells from the ectopic lymphoid tissue, suggesting that the splenic anti-NP B cells secrete more Ab than those from ectopic lymphoid tissue or that affinity maturation of B cells in the lipogranuloma is less efficient than in the spleen, an interpretation consistent with the lower affinity of anti-NP Abs in the sera of TMPD-treated mice vs controls (Fig. 1D) and the paucity of clonal trees. Together, these data suggest that 1) a significant portion of the low affinity serum anti-NP response in TMPD-treated mice may derive from the ectopic lymphoid tissue, and 2) affinity maturation may be defective in the ectopic lymphoid tissue, i.e., high affinity B cells may enjoy less of a competitive advantage over lower affinity cells in ectopic lymphoid tissue than in authentic secondary lymphoid tissues. We speculate that reduced affinity maturation in the lipogranulomas might reflect an absence of follicular dendritic cells in view of the lack of FDC-M1^- staining (7).

Further studies will be necessary, however, to determine whether the low affinity of serum anti-NP Abs in TMPD-treated mice is due to their production in ectopic lymphoid tissue or whether it is a systemic effect of TMPD treatment.

The presence of oligo-clonal B cell populations in individual lipogranulomas, lack of shared H chain sequences between lipogranulomas and spleen and between individual lipogranulomas from the same mouse, expression of activation-induced cytidine deaminase, and the presence of circular DNA intermediates generated during active class switch recombination (D. C. Nacionales et al., submitted), as well as the presence of proliferating B and T cells in these structures lead us to conclude that TMPD-induced ectopic lymphoid tissue is a site of germinal center-like cognate T-B interactions. However, the lipogranulomas may not be true germinal centers and instead could be more analogous to the previously reported extrafollicular sites of Ag-driven somatic hypermutation of rheumatoid factor B cells (24). Recently, ectopic lymphoid tissue was also found to express activation-induced cytidine deaminase in the salivary glands of patients with Sjögren’s syndrome (25), supporting the idea that this may be a general feature of ectopic lymphoid tissue in a variety of locations.

The formation of ectopic lymphoid tissue is strongly associated with autoimmunity and autoantibody production in a variety of disorders (2), including the rheumatoid synovium (21, 26), salivary glands in Sjögren’s syndrome (27), thymus in myasthenia gravis (19), and the thyroid in Hashimoto’s thyroiditis (28). In several examples of organ-specific autoimmune disease, autoreactive B cells have been found within ectopic lymphoid tissue in the target tissues. We have shown recently that anti-RNP autoantibody-producing B cells are enriched in ectopic lymphoid tissue of TMPD-treated mice, strongly suggesting that this may be a site where autoreactivity may develop preferentially (D. C. Nacionales et al., submitted). It will be of interest to determine where the APCs responsible for activating autoreactive T cells acquire self-Ags, as the present data indicate that APCs from remote (e.g., s.c.) locations are capable of homing to ectopic lymphoid tissue located within the peritoneum. The role of chemokines, such as CXCL19, CXCL21, and CXCL13, expressed at high levels in the ectopic lymphoid tissue (7) in establishing autoantibody production in ectopic sites remains to be determined. Finally, it will be of interest to see whether therapy aimed at disrupting the formation of ectopic lymphoid tissue will prevent the development of lupus in TMPD-treated mice.

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**Disclosures**

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**References**


