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Maintenance of Anti-Sm/RNP Autoantibody Production by Plasma Cells Residing in Ectopic Lymphoid Tissue and Bone Marrow Memory B Cells

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Although ectopic lymphoid tissue formation is associated with many autoimmune diseases, it is unclear whether it serves a functional role in autoimmune responses. 2,6,10,14-Tetramethylpentadecane causes chronic peritoneal inflammation and lupus-like disease with autoantibody production and ectopic lymphoid tissue (lipogranuloma) formation. A novel transplantation model was used to show that transplanted lipogranulomas retain their lymphoid structure over a prolonged period in the absence of chronic peritoneal inflammation. Recipients of transplanted lipogranulomas produced anti-U1A autoantibodies derived exclusively from the donor, despite nearly complete repopulation of the transplanted lipogranulomas by host lymphocytes. The presence of ectopic lymphoid tissue alone was insufficient, as an anti-U1A response was not generated by the host in the absence of ongoing peritoneal inflammation. Donor-derived anti-U1A autoantibodies were produced for up to 2 mo by plasma cells/plasmablasts recruited to the ectopic lymphoid tissue by CXCR4. Although CD4+ T cells were not required for autoantibody production from the transplanted lipogranulomas, de novo generation of anti-U1A plasma cells/plasmablasts was reduced following T cell depletion. Significantly, a population of memory B cells was identified in the bone marrow and spleen that did not produce anti-U1A autoantibodies unless lipogranulomas, de novo generation of anti-U1A plasma cells/plasmablasts was reduced following T cell depletion. Significantly, a population of memory B cells was identified in the bone marrow and spleen that did not produce anti-U1A autoantibodies unless stimulated by LPS to undergo terminal differentiation. We conclude that 2,6,10,14-tetramethylpentadecane promotes the T cell–dependent development of class-switched, autoreactive memory B cells and plasma cells/plasmablasts. The latter home to ectopic lymphoid tissue and continue to produce autoantibodies after transplantation and in the absence of peritoneal inflammation. However, peritoneal inflammation appears necessary to generate autoreactive B cells de novo.

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Lymphoid neogenesis, the formation of ectopic (tertiary) lymphoid tissue in response to chronic inflammation (1), is associated with autoantibody production in Sjögren’s syndrome, rheumatoid arthritis, myasthenia gravis, and other diseases (2–4). Although many features of secondary lymphoid tissue development are recapitulated (5), it is unclear whether ectopic lymphoid tissue participates directly in generating autoreactive B cells, indirectly as a reservoir for Ab-secreting cells, or both.

Approximately 3 mo after i.p. exposure of non–lupus-prone mice to the hydrocarbon oil 2,6,10,14-tetramethylpentadecane (TMPD; pristane), “lipogranulomas” (ectopic lymphoid tissue) form, and the mice develop high levels of IgG lupus-associated autoantibodies (e.g., anti-Sm/RNP and DNA) and nephritis (6, 7). The production of these class-switched autoantibodies requires T cells (8). In contrast, mice treated i.p. with mineral oil (a mixture of hydrocarbons) develop ectopic lymphoid tissue, but not lupus. The inflammatory response to TMPD (but not mineral oil) is maintained chronically by a vicious circle of IFN-I production, stimulating the expression of CCL2 and other IFN-I–inducible chemokines, which recruit additional IFN-I–producing cells into the lipogranulomas (9). T and B lymphocytes and dendritic cells are recruited to the inflamed peritoneum by the chemokines CCL19, CCL21, and CXCL13, and the ectopic lymphoid tissue develops distinct T cell/dendritic cell and B cell zones (10). B cells in the ectopic lymphoid tissue exhibit some features of the germinal center response, including proliferation, class-switch recombination, and expression of activation-induced cytidine deaminase (8).

After primary immunization with the exogenous Ag NP-KLH, Ag-specific B and T lymphocytes home to TMPD-induced ectopic lymphoid tissue and proliferate. In addition, class-switched, NP-specific Ig is produced by cells residing in the lipogranulomas (11). Similarly, IgG anti-RNP (U1A) autoantibody-secreting cells (ASCs) are highly enriched in TMPD-induced ectopic lymphoid tissue compared with spleen (8). However, the origin of these cells and their relationship to the persistent anti-U1A autoantibody levels in the serum are unknown.

Long-term serological memory can be maintained by long-lived plasma cells (PCs), normally found in the bone marrow (BM), which may continue to secrete Abs for many years (12). Alternatively, serological memory may be maintained by T cell–de-
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Detection of autoantibodies by immunoprecipitation

The presence of anti-Sm/RNP autoantibodies was confirmed by immunoprecipitation of 58S-labeled cellular proteins and analyzed on a 12.5% SDS-polyacrylamide gel, as described (6).

RT-PCR

RT RNA was precipitated with isopropanol and the pellet washed with cold 75% (v/v) ethanol and resuspended in diethyl pyrocarbonate-treated water. A total of 1 μg RNA was reverse transcribed to cDNA, using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Then 1 μl cDNA was added to the PCR mixture containing PCR buffer, 2.5 mmol/l MgCl2, 400 μmol/l 2′-deoxynucleoside 5′-triphosphate, 0.025 U Taq DNA polymerase (Invitrogen), and 1 μmol/l each of forward and reverse primers in a 20-μl volume. Primers were as follows: CXCL12 forward, 5′-ATG AGT AGT CTG AGC CTC C-3′, and reverse, 5′-GAG CCC TCT TTC TTT CC-3′; CXCL13 forward, 5′-ATG AGG CTC AGC ACA GCA AC-3′, and reverse, 5′-CCA TTC GGC ACC AGG ATT CAC-3′; 18S forward, 5′-GGGCTACACATCAGAGAA3′, and reverse, 5′-GCTGAGAATCCGCGGT-3′. Reactions were heated for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min, extension at 72°C for 1 min, and a final extension of 72°C for 10 min in a PTC-100 programmable thermal controller (Bio-Rad, Hercules, CA). PCR primers were synthesized by Invitrogen.

Quantitative PCR

Gene expression was quantified by real-time PCR. A total of 1 μl cDNA was added to a mixture containing 3.75 mmol/l MgCl2, 1.25 mmol/l dNTP mixture, 0.025 U AmpliTaq Gold, SYBR Green dye (Applied Biosystems, Foster City, CA), and optimized concentrations of specific forward and reverse primers in a final volume of 20 μl. CXCL12 primers were as follows: forward, 5′-TGG TCT CTT CTT GCC GCC TCC A-3′, and reverse, 5′- GTG CCG TCA GCC TAC AGA GTG-3′. The 18S cDNA primers were as above. Amplification conditions were 95°C (10 min), followed by 45 cycles of 94°C (15 s), 60°C (25 s), and a final extension at 72°C for 8 min, using a DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research, St. Bruno, QC, Canada). Transcripts were quantified using the comparative (2−ΔΔCT) method.

T cell depletion

CD4 T cells were depleted as described (17, 18). Briefly, either anti-CD4 Ab ( GK1.5 hybridoma, 500 μg) or rat anti-human IL-4 ( IgG2b isotype control; Schering Plough Biopharma, Palo Alto, CA) was injected i.p. Donor mice received GK1.5 or rat anti-human IL-4 Ab 4 d prior to transplantation. Depletion lasted up to 7 d and was confirmed by flow cytometry. Recipients received GK1.5 or rat anti-human IL-4 Ab at the time of transplantation and weekly thereafter. After 35 d, splenocytes, lipogranulomas, BM, and blood were harvested, and depletion of CD4 T cells was confirmed by flow cytometry.

ELISPOT assay for anti-RNP ASCs

The production of anti-U1A (a subset of anti-RNP) autoantibodies in the ectopic lymphoid tissue was examined by ELISPOT assay, as previously described (8).

Immunohistochemistry

Femurs were obtained from BALB/c mice treated 6 wk earlier with TMPD and from untreated controls. The bones were fixed in 10% neutral buffered formalin for 1 h, rinsed in water for 10 min, then decalcified in Rapid Cal Immuno Decal Solution (BBC Biochemicals, Mt. Vernon, WA) for 3 h and rinsed again in water for 15 min. Specimens were embedded in paraffin, and 4-μm paraffin sections were cut, placed on plus slides, and dried for 2 h at 60°C. The slides were then placed in a Ventana Benchmark automated immunostainer and deparaffinized. Heat-induced epitope retrieval was performed with Ventana’s CC1 retrieval solution for 30 min at 95–100°C. Preincubated peroxidase-conjugated goat anti-mouse κ-l Chain Abs (Ventana Medical Systems, Tucson, AZ) were applied to the tissue for 8 min at 37°C. The presence of cells with intracellular L chain was visualized using the ultraView DAB Detection Kit (Ventana). Slides were counterstained with Ventana Hematoxylin.

In vivo inhibition of CXCR4

CXCR4 inhibition was performed as previously described (18). Briefly, TMPD-treated anti-U1A+ mice received either 10 mg/kg i.p. AMD3100 (Sigma-Aldrich) in sterile PBS every 24 h or PBS alone. At 15 h after the

Materials and Methods

Mice

Female 6-wk-old C57BL/6, BALB/c/J, CB.17, and T cell transgenic C.57BL/6J-Tg (DO11.10)10Dlo/J (DO11.10) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in barrier cages. At 2 mo of age, C57BL/6, BALB/c/J, CB.17, and DO11.10 mice received 0.5 ml i.p. TMPD (Sigma-Aldrich, St. Louis, MO) or mineral oil (Harris Teeter, Matthews, NC) or were left untreated. Lipogranulomas were harvested for transplantation 3 mo later. These studies were approved by the Institutional Animal Care and Use Committee.

Lipogranuloma transplantation

For transplantation, TMPD-induced lipogranulomas were harvested from mice producing anti-U1A Abs (confirmed by ELISA). Recipient mice underwent an upper midline laparotomy beginning at the midabdomen and terminating superiorly at the xiphoid process. The harvested donor lipogranulomas were then transplanted onto the lateral aspect of the peritoneal surface of the right and left costodiaphragmatic junctions, using a 6-0 polypropylene monofilament suture. The midline laparotomy was reapproximated with interrupted s. monofilament sutures and the overlying skin secured with surgical wound clips. Mice received 1 ml physiological saline for resuscitation at completion of transplantation. When indicated, mice also received lipogranuloma tissue s.c. or i.p. without any suture to hold it in place. Sham procedures were carried out with a midline laparotomy and placement of a 6-0 polypropylene monofilament suture on the lateral aspect of the peritoneal surface of the right and left costodiaphragmatic junctions. Sham-transplanted mice also received normal saline at the termination of the procedure.

Flow cytometry

Cell suspensions from transplanted lipogranulomas or recipient spleens were analyzed using annexin V plus 7-aminocinomycin D (7-AAD) staining (Apopototic Cell Kit; BD Biosciences, San Jose, CA). T cells were analyzed with anti-CD3, anti-CD4, anti-B220, anti-CD11b, anti-CD25, anti-IgM, and anti-IgM Abs (BD Biosciences), and anti-Foxp3 Abs (eBioscience, San Diego, CA). DO11.10 T cells were identified using anti-CD3, anti-CD4, anti-CD25, anti-CD11b, anti-CD25, and anti-Foxp3 Abs (BD Biosciences), and optimized concentrations of specific forward and reverse primers in a final volume of 20 μl. CXCL12 primers were as follows: forward, 5′-TGG TCT CTT CTT GCC GCC TCC A-3′, and reverse, 5′- GTG CCG TCA GCC TAC AGA GTG-3′. The 18S cDNA primers were as above. Amplification conditions were 95°C (10 min), followed by 45 cycles of 94°C (15 s), 60°C (25 s), and a final extension at 72°C for 8 min, using a DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research, St. Bruno, QC, Canada). Transcripts were quantified using the comparative (2−ΔΔCT) method.

Anti-U1A (RNP) ELISA

The ELISA was carried out as described previously, using 6-His-tagged recombinant U1A protein expressed in E. coli (5 μg/ml) as Ag (8). Serum samples were tested at a 1:250 dilution, followed by incubation with alkaline phosphatase-labeled goat anti-mouse IgG (1:1000 dilution) or biotinylated anti-IgG2a, IgG2b ( IgG2c), or IgM (BD Biosciences; 1 h at 22°C); a 45-min incubation with NeutrAvidin (Southern Biotechnology, Birmingham, AL); and development with p-nitrophenyl phosphate substrate (Sigma-Aldrich). OD at 405 nm (OD405) was read using a VersaMax Microplate Reader ( Molecular Devices Corporation, Sunnyvale, CA).

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viable tissue, and provides a platform for the study of the immune response to the Sm/RNP autoantigen (anti-U1A autoantibodies) in the tissue that is the source of the autoantibodies.

We found that ongoing peritoneal inflammation was required to generate new anti-U1A B cells. PCs/PBs producing anti-U1A autoantibodies accumulated in the ectopic lymphoid tissue induced by TMPD, but the lipogranulomas were nearly devoid of anti-U1A memory B cells. Transplantation of lipogranulomas into the noninflamed peritoneum of another mouse did not abolish autoantibody production, but anti-U1A autoantibodies were exclusively of donor origin. Unexpectedly, the BM of mice treated 3–4 mo earlier with TMPD proved to be a major reservoir of anti-U1A memory B cells but contained very few anti-U1A PCs/PBs.

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last AMD3100 treatment, mice were sacrificed and lipogranulomas were excised and transplanted into untreated recipients, as described above. In some experiments, TMPD-treated mice were injected daily with either AMD3100 or PBS for 3 d. The mice then received BrdU (0.2 mg in PBS i.p. twice daily for 2 d). At 12 h after the final BrdU injection, the mice were sacrificed and spleen and lipogranulomas were harvested. BrdU incorporation into IgM CD138 PCs was detected by intracellular staining using an allophycocyanin-conjugated anti-BrdU Ab (BD Biosciences) and analyzed by flow cytometry.

**Results**  
**Transplanted lipogranulomas become revascularized and are functional**

Ag-specific B and T lymphocytes, including autoantibody-producing cells, home to TMPD-induced lipogranulomas (11). About 10–15% of the CD4+ T cells and CD19+B cells residing in this ectopic lymphoid tissue exhibited an activated (CD69+) phenotype in contrast to the low percentage of activated lymphocytes in spleen cells from the same mice (Fig. 1A). Further characterization of the CD4+ and CD8+ T cells in the lipogranulomas revealed that the majority (80–90%) were CD44hiCD62Lneg memory cells (Supplemental Fig. 1A). A high percentage of BM CD4+ T cells also exhibited a memory phenotype, as reported previously (19), whereas the phenotypes of splenic T cells were more diverse.

We next asked whether this ectopic lymphoid tissue can function outside the setting of chronic TMPD-induced peritoneal inflammation by transplanting lipogranulomas from TMPD-treated mice seropositive for anti-U1A autoantibodies into non–TMPD-treated (anti-U1A−) recipients. After 35 d, the transplanted lipogranulomas had an appearance similar to that of pretransplant ectopic lymphoid tissue when stained with hematoxylin & eosin (Fig. 2A). The transplanted tissue adhered tightly to the mesothelial surface of the peritoneum overlying the abdominal musculature and was vascularized, as determined by the distribution of i.v. injected Evans Blue dye (EBD) (Fig. 2B). Blue staining of the transplanted lipogranulomas confirmed that blood vessels in the transplanted ectopic lymphoid tissue (8) became connected to the host’s circulation. To verify that the cells in the transplanted lipogranulomas remained viable, a single-cell suspension was stained with annexin V and 7-AAD, markers of apoptosis and necrosis, respectively, and the total cell population was analyzed by flow cytometry (Fig. 2C). Approximately 50% of the total cells isolated from transplanted lipogranulomas were annexin V− 7-AAD−, similar to the percentage of live cells found in pretransplant lipogranulomas (57% annexin V− 7-AAD−) and mineral oil–induced lipogranulomas (54% annexin V− 7-AAD−). Thus, not only were the lipogranulomas revascularized after transplantation, but they also contained viable cells similar in number to those found in pretransplant lipogranulomas.

By flow cytometry, the cellular composition of transplanted lipogranulomas was similar to that of pretransplant lipogranulomas and recipient spleen (Fig. 2D). At day 35, the transplanted lipogranulomas contained 28% CD4 T cells and 46% B cells, versus 24% and 51%, respectively in non-transplanted lipogranulomas (Fig. 2D). However, lymphocytes from the transplanted lipogranulomas did not express CD69 (Fig. 1B). Similarly, the percentages of CD11b+B220+ cells (monocytes) in the transplanted
and pretransplant lipogranulomas were similar (11% and 10%, respectively), but greater than the percentage in the spleen (3.4%) (Fig. 2D). These data suggest that the composition of pre- and posttransplantation ectopic lymphoid tissue is similar, although the transplanted lipogranulomas lack a population of activated (CD69+) lymphocytes.

**Production of anti-U1A autoantibodies by transplanted lipogranulomas**

Lipogranulomas contain ASCs detectable using ELISPOT assays (8). To evaluate the functionality and ultimate fate of these cells following transplantation of lipogranulomas from anti-U1A+ TMPD-treated mice, serum levels of IgG anti-U1A autoantibodies were determined in the transplant recipients at 0, 7, 14, and 28 d. Serum anti-U1A activity was detectable by ELISA in mice receiving TMPD lipogranulomas starting at day 7–14 posttransplantation and increased up to 28 d posttransplantation (Fig. 3A). However, anti-U1A levels in sera from the transplant recipients were lower than those in the donor mice, probably reflecting the transplantation of only a small number of lipogranulomas (two to five per recipient) and failure of donor PCs/PBs to expand after transplantation (Fig. 3A). These autoantibodies also could be detected in sera of the recipients by immunoprecipitation (Fig. 3B). In contrast, mice transplanted with mineral oil (anti-U1A−) lipogranulomas, mice transplanted s.c. with TMPD-induced lipogranulomas from anti-U1A− mice, and sham-operated mice did not develop detectable levels of anti-U1A by 28 d (Fig. 3A).

To determine whether autoantibody production by the transplanted ectopic lymphoid tissue was affected by chronic peritoneal inflammation, recipient mice were pretreated with TMPD or mineral oil 2 wk prior to transplantation with lipogranulomas from anti-U1A+ donors. In comparison with untreated controls, the strong inflammatory response induced 2 wk after TMPD treatment, which is characterized by chronic IFN-I production (20), did not increase serum autoantibody levels in the recipient mice (Fig. 3C). Sham-transplanted mice also were pretreated with TMPD to verify that pretreatment with TMPD did not induce anti-U1A autoantibody production independently of the transplanted ectopic lymphoid tissue. These control mice did not produce anti-U1A autoantibodies. Anti-U1A Abs remained detectable in the sera of mice transplanted with lipogranulomas from TMPD-treated donors up to 60 d afterward (Fig. 3C). In contrast, the TMPD-pretreated sham mice began to produce anti-U1A by day 60 posttransplantation (80 d after TMPD pretreatment), consistent with previous observations that TMPD-treated mice develop an anti-Sm/RNP response at ~3 mo posttreatment (6). Despite producing autoantibodies, the transplanted mice failed to develop proteinuria (Fig. 3D). These data suggested that the transplanted lipogranulomas contained PCs/PBs capable of maintaining serum anti-U1A autoantibody levels over an extended period.

**Recipient T cells enter the transplanted lipogranulomas**

To examine the role of T cells in the production of autoantibodies in the recipients, we transplanted anti-U1A+ lipogranulomas from BALB/c mice into BALB/c CD4+ T cell transgenic DO11.10 mice. Unexpectedly, serum anti-U1A autoantibody levels were higher in DO11.10 recipients than in wild-type controls (p = 0.02, Mann–Whitney U test; Fig. 4A). Using an Ab against the transgenic T cells (KJ1-26), we found that by 35 d after transplantation, donor lipogranulomas were repopulated with numerous recipient T cells (Fig. 4B, 4C). Approximately 75–80% of the CD4+ T cells in the transplanted lipogranulomas were of recipient (transgenic) origin, a percentage similar to that in the spleen. The transplanted lipogranulomas expressed mRNA for the T cell–attractive chemokine CXCL21 (Fig. 4D), which may mediate the influx of recipient T cells into the transplant (21). These data suggest that
after transplantation, and the percentages of recipient and spleen were harvested from DO11.10 mice 35 d after transplanting wild-type lipogranulomas into wild-type CB.17 recipients. To assess for the possibility that the recipient's regulatory T cells might reduce anti-U1A autoantibody production, because CD4+CD25+Foxp3+ regulatory T cells can downregulate autoantibody production (22). Consistent with that possibility, numbers of these cells in the ectopic lymphoid tissue increased posttransplantation. *p = 0.007, Mann–Whitney U test. (D) BALB/c lipogranulomas were transplanted into BALB/c recipients, and cDNA from transplanted lipogranulomas (Lipogran) and recipient spleen was tested for CXCL21 expression by RT-PCR and compared with 18S rRNA expression (representative of four experiments). (E) T cells with a regulatory phenotype (CD4+CD25+Foxp3+) were analyzed from pretransplant (BALB/c) lipogranulomas and from lipogranulomas 35 d posttransplantation into BALB/c recipients by staining for surface markers CD4 and CD25 and the intracellular marker Foxp3 (flow cytometry, n = 4). The percentage of T cells with a regulatory phenotype increased posttransplantation. *p = 0.02, Mann–Whitney U test.

Autoantibodies are derived exclusively from donor B cells

We examined whether the recipient's B lymphocytes also could enter the transplanted ectopic lymphoid tissue by transplanting anti-U1A lipogranulomas from allototype congenic CB.17 (Ighb) donors into BALB/c (Igha) recipients. We took advantage of the fact that anti-U1A autoantibodies induced by TMPD are predominately IgG2b. Instead of IgG2a, the CB.17 strain expresses IgG2a (also termed IgG2c). Using an IgG2a allototype-specific anti-U1A ELISA, we found that all of the serum anti-U1A autoantibodies in BALB/c mice transplanted with CB.17 lipogranulomas were of donor (CB.17) origin (Fig. 5A). The level of serum IgG2a (BALB/c origin) anti-U1A autoantibodies was no different from that of sham-transplanted mice. In contrast, IgG2a (CB.17 origin) anti-U1A autoantibody levels increased significantly following transplantation, indicating that the anti-U1A autoantibodies were produced exclusively by donor-derived PCs/PBs.

Because T cells populating the transplanted lipogranulomas were primarily of recipient origin, we next examined whether B cells populating the lipogranulomas posttransplantation were of donor or recipient origin. The presence of donor and recipient memory B cells residing in the transplanted lipogranulomas. (A) DO11.10 or BALB/c mice received U1A+ lipogranulomas from TMPD-treated BALB/c mice (n = 6) or were sham operated (Sham-DO11.10). Sera were collected at days 0, 7, 14, 28, and 35 following surgery, and IgG anti-U1A Abs were assessed by ELISA. (B) Lipogranulomas and spleen were harvested from DO11.10 mice 35 d after transplantation, and the percentages of recipient (CD4+KJ1-26+) T cells were determined by flow cytometry (gated on lymphocytes and CD4+ cells). (C) BALB/c lipogranulomas were transplanted into DO11.10 recipients, and transgenic T cells (KJ1-26+) as a percentage of total CD4+ T cells were assessed in lipogranulomas 35 d after transplantation and compared with the percentage of pretransplant lipogranulomas. *p = 0.007, Mann–Whitney U test. (D) BALB/c lipogranulomas were transplanted into BALB/c recipients, and cDNA from transplanted lipogranulomas (Lipogran) and recipient spleen was tested for CXCL21 expression by RT-PCR and compared with 18S rRNA expression (representative of four experiments). (E) T cells with a regulatory phenotype (CD4+CD25+Foxp3+) were analyzed from pretransplant (BALB/c) lipogranulomas and from lipogranulomas 35 d posttransplantation into BALB/c recipients by staining for surface markers CD4 and CD25 and the intracellular marker Foxp3 (flow cytometry, n = 4). The percentage of T cells with a regulatory phenotype increased posttransplantation. *p = 0.02, Mann–Whitney U test.
we harvested at day 35, and the B cells (B220+) were determined by flow cytometry. As shown in Fig. 6C, CD4+ Ighb from CB.17 (n = 6). Sera were collected at days 0, 7, 14, 28, and 35, and IgG2a/IgG2c (TMPD-IgG2c) or IgG2a/IgG2a (TMPD-IgG2a) anti-U1A Abs were assessed by ELISA. IgG2a anti-U1A Abs also were measured in control, sham-transplanted mice (Sham-IgG2c). (B and C) Transplanted lipogranulomas or nontransplanted lipogranulomas from CB.17 mice were harvested at day 35, and the B cells (B220+) were stained for recipient (IgM+) or donor (IgM+) allotypes (flow cytometry). A representative plot shows the percentage of allotype-specific B cells in transplanted lipogranulomas before and after transplantation into BALB/c recipients. Data are representative of two experiments. *p = 0.02, Mann–Whitney U test. (D) cDNA from a BALB/c transplanted lipogranuloma (Lipogran) or spleen from a recipient mouse was tested for CXCL13 expression (RT-PCR) and compared with 18S rRNA (representative of four experiments).

We administered GK1.5 mAbs weekly to U1A+ TMPD-treated mice. Peripheral blood CD4 counts were monitored every 7 d to verify depletion of all CD4+ cells (data not shown). After 35 d of weekly GK1.5 treatment, lipogranulomas and spleen were harvested, and the presence of live CD4+ T cells was determined by flow cytometry. As shown in Fig. 7A, the lipogranulomas and spleen did not contain any CD4+ T cells. In contrast to the transplanted mice (Fig. 6), in nontransplanted mice depletion of CD4+ T cells caused a 60% decrease in the levels of serum IgG anti-U1A autoantibodies between days 0 and 35 (Fig. 7B, left). However, despite substantially decreasing after T cell depletion, serum IgG anti-U1A remained significantly higher in GK1.5-treated mice than the background levels in non–TMPD-treated controls (Fig. 7B, right). This finding may reflect a population of anti-U1A–secreting cells similar or identical to those observed in the transplanted lipogranulomas (Fig. 6), although we cannot exclude the possibility that the residual autoantibody levels might have been due to incomplete depletion of CD4+ T cells in other sites, such as omentum, mesenteric lymph node, parathyroid lymph node, or BM.

Anti-U1A memory B cells are present in the BM and spleen

IgG memory cells are a hallmark of the germinal center reaction, and the maturation of these cells into PCs/PBs generally is dependent on CD4+ T cells (15). This observation suggested that some anti-U1A autoantibodies could have been produced by PCs/PBs derived from the activation of switched memory B cells. Murine memory B cells, but not PCs, can be stimulated by LPS to secrete Ab in vitro (16, 17). To look for anti-U1A memory cells, B cells from the lipogranulomas, spleen, or BM of TMPD-treated, anti-U1A+ mice were cultured in the presence or absence of LPS (5 μg/ml) followed by assay by ELISPOT of the numbers of

the donor and recipient mice, using mAb GK1.5. Anti-U1A+ donor mice were treated with either GK1.5 or a rat anti-human IL-4 Ab control for 4 d prior to surgery. In mice receiving GK1.5, nearly all CD4+ T cells were eliminated from the donor lipogranulomas compared with the control (Fig. 6A). Recipient mice were treated with GK1.5 or the irrelevant control Ab (rat anti-human IL-4) at the time of surgery and continued to receive weekly treatments up to 35 d postsurgery. CD4+ T cells remained depleted in the peripheral blood of the GK1.5-treated recipient mice throughout the 35-d duration of this experiment, whereas a control Ab had no effect (Fig. 6B). After 35 d, lipogranulomas and spleens were excised from mice that received GK1.5 or control Ab, and the numbers of viable (CD4+, Sytox blue−) cells were determined by flow cytometry. As shown in Fig. 6C, CD4+ T cells were undetectable in the GK1.5-treated mice. The numbers of CD138+CD44+ PCs/PBs decreased substantially in the spleen and lipogranulomas of the GK1.5-treated mice (Fig. 6D; p = 0.016, Mann–Whitney U test, for both spleen and lipogranulomas). In contrast, depletion of CD4+ cells had little effect on the levels of serum IgG anti-U1A autoantibodies in transplanted mice at 35 d (Fig. 6E). As the half-life of IgG Abs in an adult mouse is ~3 wk (24), the unchanged autoantibody levels suggest that serum anti-U1A autoantibodies in transplanted mice were derived, at least in part, from a population of PCs/PBs that was maintained over a period of 5 wk independently of cognate T–B cell interaction.

Effect of CD4+ T cell depletion in nontransplanted mice

We next examined whether depleting CD4+ T cells had any effect on anti-Sm/RNP autoantibody production in nontransplanted TMPD-treated mice. Anti-Sm/RNP and anti-U1A autoantibodies cannot be induced by TMPD in nude mice or TCR-deficient mice (8, 25). However, the role of T cells in maintaining autoantibody production once it has been established has not been examined.
anti-U1A–secreting cells. In the spleen, but not the lipogranulomas, the number of IgG anti-U1A spots increased significantly in the presence of LPS, consistent with the presence of a switched memory B cell population (Fig. 7C). Unexpectedly, unstimulated BM from TMPD-treated mice did not contain IgG anti-U1A–producing cells, whereas the number of spots increased dramatically after LPS stimulation. In contrast, LPS treatment of spleen or BM from control (non–TMPD-treated) mice did not stimulate the secretion of anti-U1A Abs detectable by ELISPOT assay (Fig. 7C). Because naive B cells can be stimulated by LPS to produce T cell–independent IgG3 Abs (26–28), IgG3 spots were compared with total IgG to verify that most of the anti-U1A was of T cell–dependent isotypes (i.e., IgG1, IgG2a, and/or IgG2b) (Fig. 7D). These data indicate that the BM and spleen of TMPD-treated mice, but not control (non–TMPD-treated) mice, contain a population of anti-U1A B cells that do not secrete autoantibodies at rest but can be stimulated to secrete anti-U1A by TLR4 ligation. These are likely to represent memory B cells and/or early PBs. Of interest, although flow cytometry suggests that the BM, spleen, and lipogranulomas all contained B cells phenotypically consistent with memory B cells (B220\(^+\)CD38\(^+\)IgM\(^-\)); Supplemental Fig. 1B), the memory cells in lipogranulomas could not be activated to make anti-U1A by LPS and therefore may not have participated in autoantibody production in the mice transplanted with lipogranulomas. When T cells were depleted in nontransplanted anti-U1A+ mice, using GK1.5 Abs, the number of anti-U1A spots in the lipogranulomas and spleen decreased by ∼50% (Fig. 7E). Thus, T cells appear to help maintain the numbers of anti-U1A PCs/PBs (Fig. 7E), as well as at least a portion of the serum anti-U1A autoantibody levels (Fig. 7B). This finding could reflect an effect on the formation of new anti-U1A PCs/PBs, on the survival of these cells, or both.

TMPD treatment depletes PCs/PBs from the BM

A striking and unexpected observation of the ELISPOT experiments (Fig. 7C, 7E) was the absence of spontaneous anti-U1A–secreting cells in the BM, a major site for the accumulation of long-lived PCs (29). To examine whether the absence of PCs/PBs was unique to autoantibody-producing cells or was a more general phenomenon, we determined the effects of TMPD treatment on the total number of CD138\(^+\)IgM\(^-\)B220\(^-\) PCs/PBs in the BM of untreated or TMPD-treated (after 3–4 mo) wild-type mice. As

![Diagram](image-url)

**FIGURE 6.** Serum anti-U1A Abs in transplanted mice persist after T cell depletion. (A) Depletion of T cells. U1A\(^+\) lipogranulomas were isolated from TMPD-treated mice injected 4 d earlier with the CD4 T cell–depleting mAb GK1.5 (\(n = 5\), right panel) or rat anti-human IL-4 Ab (Control, \(n = 4\), left panel). Flow cytometry of single-cell suspensions of lipogranuloma cells from the donor mice, using anti-B220 and anti-CD4 Abs, verified nearly complete depletion of CD4\(^+\) T cells in the GK1.5-treated mice. In contrast, treatment with an isotype control (rat anti-human IL-4 mAb) had no effect. (B) Recipient mice (which were transplanted with lipogranulomas from GK1.5-treated or rat anti-human IL-4 Ab anti-U1A\(^+\) donors) were treated at day 0 and every 7 d thereafter with either GK1.5 or control (rat anti-human IL-4) Abs. CD4\(^+\) T cell depletion was monitored in peripheral blood every 7 d by flow cytometry. (C) Lipogranulomas and spleen were excised from recipient mice 35 d after transplantation, and CD4 T cells were examined by flow cytometry. Shaded, GK1.5-treated recipients; open, control Ab-treated recipients. (D) PCs (CD44\(^+\)CD138\(^+\)) from recipient spleen and transplanted lipogranulomas were analyzed at day 35 by flow cytometry after treating both the donor and the recipient with GK1.5 or control Ab. *\(p = 0.01\), Mann–Whitney U test. (E) Sera were collected from either GK1.5- or control Ab–treated recipients at days 0, 7, 14, 28, and 35 posttransplantation. Serum IgG anti-U1A levels were assessed by ELISA (representative of two experiments).
shown in Fig. 8A, numbers of BM PCs/PBs were substantially lower in TMPD-treated wild-type mice than in non–TMPD-treated mice. Decreased BM PCs/PBs also were apparent following immunohistochemical staining of the BM for intracellular k-L chain+ cells (Fig. 8B). Because increased IFN-I levels cause lymphopenia in the BM (30), we examined whether IFN-I production induced by TMPD caused the decreased numbers of BM PCs. However, in the BM of TMPD-treated IFNAR−/− mice, the percentage of CD138+IgM+ IgG2a B220− PCs was comparable to the percentage in wild-type TMPD-treated mice or from non–TMPD-treated controls (No TMPD Rx). B cells were negatively selected and cultured in the presence or absence of LPS (5 μg/ml) for 5 d. IgG anti-U1A Ab production from cultured B cells was measured by ELISPOT. *p ≤ 0.02, **p ≤ 0.04, Mann–Whitney U test.

CXCR4 retains autoantibody-producing PCs/PBs in lipogranulomas

Inhibition of the CXCL12 receptor CXCR4 can deplete autoantibody-producing PCs in kidneys of lupus mice (35). To see whether CXCL12/CXCR4 retains PCs in ectopic lymphoid tissue, TMPD-treated anti-U1A+ mice were treated with either the CXCR4 antagonist AMD3100 or PBS before lipogranulomas were transplanted into untreated anti-U1A− recipients. After AMD3100 treatment, the numbers of CD138+CD44+ PCs/PBs in the spleen and lipogranulomas were greatly reduced (Fig. 9A). After transplanting lipogranulomas from AMD3100-treated mice, little or no autoantibody production was observed in the recipient mice for ≥35 d (Fig. 9B). However, although anti-U1A–secreting PCs/PBs were nearly absent
in the transplanted lipogranulomas from AMD3100-treated mice (Fig. 9A), lipogranulomas from AMD3100-treated and control mice contained similar percentages of total PCs/PBs 35 d after transplanting into untreated recipients (Fig. 9C), suggesting that the recipient’s PCs/PBs can home to the transplanted lipogranulomas. Thus, the lipogranulomas are likely to continue producing high levels of CXCL12 after transplantation.

To evaluate the kinetics of PB accumulation in the spleen and ectopic lymphoid tissue, TMPD-treated mice were fed BrdU for 2 d, and the percentage of BrdU+ PCs/PBs was determined 12 h later. As shown in Fig. 9D, BrdU+ PBs were present in both the ectopic lymphoid tissue (7.5% BrdU+CD138+IgM⁺ cells) and the spleen (5% BrdU+CD138+IgM⁺ cells). The numbers of PBs in these sites were unaffected by pretreatment with AMD3100 prior to BrdU labeling.

**Discussion**

Ag-specific B and T lymphocytes accumulate in ectopic lymphoid tissue induced by i.p. injection of TMPD, and ASCs can be detected readily (11). In this article, we show that when ectopic lymphoid...
tissue from anti-U1A autoantibody–positive mice was transplanted into non–TMPD-treated recipients, serum IgG anti-U1A autoantibodies derived from donor PC/PBs were produced for ≥2 mo in the absence of de novo autoantibody production by the recipients’ B cells. Unexpectedly, few cells spontaneously producing anti-U1A were found in the BM. Instead, the BM and spleen of TMPD-treated mice contained numerous nonsecreting IgG anti-U1A B cells, likely to be memory cells, which could be activated in vitro by the TLR4 ligand LPS to differentiate into ASCs. Of interest, the de novo generation of anti-U1A B cells appears to depend on ongoing peritoneal inflammation, because the transplanted ectopic lymphoid tissue did not generate anti-U1A B cells of recipient origin.

Ectopic lymphoid tissue is a reservoir for anti-U1A PCs/PBs

In addition to regaining functional capacity (revascularization and the ability to attract T and B lymphocytes) following transplantation (Figs. 2B, 4C, 5C), lipogranuloma ASCs from anti-U1A+ donors produced anti-U1A autoantibodies at levels sufficient for detection in the recipients’ serum (Fig. 3). Autoantibody levels in recipients were lower than those in donors, probably because 2–5 lipogranulomas were transplanted (versus 10–20 large lipogranulomas in each donor mouse). The lower level of autoantibodies and/or the relatively short exposure of the recipients’ kidneys to them may explain the absence of proteinuria in the recipients (Fig. 3D). In addition, the recipients lack the chronic TMPD-induced inflammatory response seen in donor mice. This response, which leads to the chronic export of inflammatory (Ly6Chis) monocytes from the BM (20), may contribute to renal damage, as inflammatory monocyte/macrophage recruitment is thought to be important in the pathogenesis of lupus nephritis (38).

The ectopic lymphoid tissue was a significant reservoir for PCs/PBs secreting IgG anti-U1A (anti-RNP) autoantibodies, with approximately double the number of U1A-specific ASCs per 100,000 B cells as the spleen (Fig. 7). Lipogranuloma PCs/PBs were the likely source of serum anti-U1A Abs in the recipients, because serum autoantibodies were not seen after transplanting lipogranulomas from donors pretreated with AMD3100 (Fig. 9B). Nevertheless, despite depletion of PCs/PBs by pretreatment with AMD3100, “normal” numbers of actively dividing PBs had reaccumulated in the ectopic lymphoid tissue 48 h after removing AMD3100 (Fig. 9D). After transplantation, serum IgG anti-U1A levels peaked at ~1 mo but were maintained for ≥2 mo (Fig. 3C). Because short-lived PCs have a lifespan of <2 wk (36, 37) and the half-life of murine IgG is <3 wk (24), some of the serum autoantibodies in the recipients of anti-U1A+ lipogranulomas may have been produced by long-lived PCs residing in ectopic lymphoid tissue niches, which, like those in the BM, may be a site where PC/PB survival factors such as APRIL are produced (34).

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It is noteworthy that pretreating the donor with the CXCR4 antagonist AMD3100 (39) not only depleted PCs from the anti-U1A+ lipogranulomas but also abolished the ability of lipogranulomas to generate serum anti-U1A autoantibodies upon transplantation (Fig. 9A, 9B). Thus, lipogranuloma B cells cannot replenish the anti-U1A PCs/PBs. The effects of AMD3100 treatment are consistent with a recent report implicating CXCR4/CXCL12 in the pathogenesis of lupus (35) and suggest that CXCR4 antagonists, already in clinical use for the treatment of HIV infection, may be useful for depleting autoreactive PCs/PBs. Indeed, both AMD3100 (40) and thalidomide, which also inhibits CXCL12/CXCR4 interactions (41), mobilize malignant PCs in the BM of multiple myeloma patients.

The BM and spleen of TMPD-treated mice contain anti-U1A memory B cells

Serological memory is maintained by long-lived PCs, which continue to produce Ig for many years, and memory B cells, which can be induced to differentiate into short- and/or long-lived PCs by T cells and/or TLR ligand stimulation (13, 42–44). Ectopic lymphoid tissue in TMPD-treated mice contained PCs/PBs producing anti-U1A autoantibodies. Because GK1.5 treatment could reduce the numbers of IgG anti-U1A ASCs in lipogranulomas and spleen by about two thirds (Fig. 7E), we suspected that some of these ASCs were generated by the terminal differentiation of memory B cells. Memory B cells, but not mature PCs, can be stimulated to produce Ab in vitro with TLR ligands, such as LPS (16). Although spontaneous anti-U1A–producing ASCs were absent in the BM, we found a population of B cells in the BM and spleen that did not secrete IgG anti-U1A autoantibodies spontaneously, but did so after LPS stimulation (Fig. 7C). These cells functionally resemble memory B cells, and B cells with a memory phenotype were detected in the BM by flow cytometry (Supplemental Fig. 1B). In contrast, the number of anti-U1A ASCs in lipogranulomas was unchanged by LPS stimulation, suggesting that although the ectopic lymphoid tissue contains PCs/PBs, few anti-U1A memory cells may be in that location or they may be incapable of responding to LPS stimulation. Retention of anti-U1A memory B cells in the BM is unlikely to involve CXCL12/CXCR4 interactions (45) because CXCL12 was greatly decreased in the BM of TMPD-treated mice but significantly increased in spleen and lipogranulomas (Fig. 8C). We hypothesize that an as yet undefined chemokine attracts or retains anti-U1A memory B cells in the BM and spleen. The BM anti-U1A B cells may represent a renewable pool of switched memory cells capable of developing into ASCs that can home to ectopic lymphoid tissue in response to CXCL12.

It is of interest that the number of PCs in transplanted lipogranulomas decreased substantially when CD4+ T cells were depleted with GK1.5 mAb (Fig. 6). PCs/PBs are attracted to BM survival niches by stromal cells producing CXCL12, whereas their survival within these niches is critically dependent on the interaction of PC B cell maturation Ag with APRIL (34, 46, 47). T cells are not required to maintain PBs in these survival niches (12, 13). Similarly, the survival of memory B cells depends on factors other than cognate T cell help (48). In contrast, in many (but not all) cases the generation of PBs from switched memory B cells requires the interaction of T cell CD40L with B cell CD40 and other signals (15, 48–50). Together, the reduction of PCs/PBs in the ectopic lymphoid tissue following GK1.5 treatment and the rapid reappearance of BrdU+ PBs in the lipogranulomas following AMD3100 treatment (Fig. 9D) suggest that the presumptive memory B cells in the BM and spleen may mature into PBs that migrate to the ectopic lymphoid tissue in response to the high levels of CXCL12 produced there (Fig. 8C). Our data are consistent with previous reports that long- and short-lived PCs home to the inflamed kidneys and spleen in NZB/W mice (51). Autoantibody-producing PCs also accumulate in ectopic lymphoid tissue in patients with Sjo¨gren’s syndrome, rheumatoid arthritis, or myasthenia gravis (4, 20, 34).

Our data are consistent with previous reports that the BM contains memory B cells specific for exogenous Ags, serving as
a site of Ag-driven and T cell–dependent differentiation of memory B cells into ASCs (13, 42). Of note, the BM is a site of CD4+ memory T cell accumulation (19) as well, suggesting that the memory B cells also found there might receive cognate help and begin the process of terminal differentiation within the BM. Remarkably, PCs/PBs were deficient in the BM of TMPD-treated mice, probably owing to inflammation-induced reductions of CXCL12 (Fig. 8C). CXCL12 recruits PCs to survival niches in the BM (52), and inflammation induced by oil adjuvants alters lymphopoiesis and granulopoiesis by altering CXCL12 expression (53, 54). Our data suggest that in addition to decreasing lymphopoiesis and increasing myeloid precursors, TMPD treatment profoundly alters the PC compartment. Whether this observation reflects the action of TNF-α and IL-1β on CXCL12 expression (54), the action of G-CSF on CXCR4 expression (55), or other, as yet unidentified, factors is under investigation. As inflammation-induced alterations of B cell homeostasis may be involved in the pathogenesis of lupus autoantibodies, the current observations may suggest new therapeutic strategies.

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The authors have no financial conflicts of interest.

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