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Murine Spleen Tissue Regeneration from Neonatal Spleen Capsule Requires Lymphotoxin Priming of Stromal Cells

Jonathan K. H. Tan1 and Takeshi Watanabe

Spleen is a tissue with regenerative capacity, which allows autotransplantation of human spleen fragments to counteract the effects of splenectomy. We now reveal in a murine model that transplant of neonatal spleen capsule alone leads to the regeneration of full spleen tissue. This finding indicates that graft-derived spleen stromal cells, but not lymphocytes, are essential components of tissue neogenesis, a finding verified by transplant and regeneration of Rag1KO spleen capsules. We further demonstrate that lymphotoxin and lymphoid tissue inducer cells participate in two key elements of spleen neogenesis, bulk tissue regeneration and white pulp organization, identifying a lymphotoxin-dependent pathway for neonatal spleen regeneration that contrasts with previously defined lymphotoxin-independent embryonic spleen organogenesis. The Journal of Immunology, 2014, 193: 1194–1203.

Autotransplantation of spleen slices depends on sections of graft tissue surviving past an initial phase of necrosis to regenerate new spleen tissue (6). However, the specific cells and molecules regulating spleen tissue neogenensis remain elusive. From a developmental perspective, events that drive embryonic lymph node (LN) organogenesis, such as lymphoid tissue organizer (LTo) and inducer cell interaction through lymphotoxin (LT)–α,β2 signaling (7), may also regulate spleen tissue development. In this pathway, stromal LTo cells expressing LT-β receptor (LTβR) engage with LT expressed on lymphoid tissue inducer (LTI) cells, leading to the activation of classic and alternative NF-κB signaling pathways, the former inducing LTI expression of adhesion molecules such as VCAM and ICAM, and the latter leading to production of homeostatic chemokines CCL19, CCL21 and CXCL13 (8). These signals increase attraction and retention of LTI in developing LN anlagen, and through ligation of CXCL13 and its corresponding CXCR5 receptor expressed on LTI (9), induce further LT expression, creating a positive feedback loop that sustains LN formation.

The extent to which spleen parallels LN development is unclear, as even between mesenteric and peripheral LN formation, differences between corresponding stromal organizers arise (10). Moreover, in gene-deficient mouse models in which LNs largely fail to develop (11–13), spleen tissue remains intact despite evidence of white pulp (WP) defects. Therefore, requirements for early LT signaling regulating LN organogenesis appear to differ with spleen. In previous transplantation models, spleen from embryonic day 15 (E15) LTα−/− mice were shown to regenerate following engraftment under the kidney capsule (14), supporting the idea that LT signaling is not required for early embryonic spleen development. Moreover, by E15 separation of spleen red pulp (RP) and WP is evident, indicating these events also occur independently of LT signaling (15).

We now demonstrate by transplant of murine neonatal spleen capsules that in contrast to bulk spleen tissue slices, stromal cells alone are capable of spleen tissue regeneration, but this process is strictly mediated by the LT pathway. Spleen capsules isolated from postnatal day 3 (D3) mice, and transplanted under the kidney capsule of adult recipients, regenerated full spleen tissue after 4 wk. This tissue displayed microarchitecture equivalent to that of native spleen with functional capacity to support Ab class switching and affinity maturation. The efficiency of tissue regeneration was donor age dependent, with capsules isolated from D3 mice developing...
more extensively than day 8 (D8) or adult grafts. Recipient age did not, however, influence regeneration potential. Graft regeneration was dependent on spleen capsule-bound stromal cells alone and not lymphocytes, because Rag1KO capsules could regenerate spleen tissue following transplant. However, by comparison with embryonic spleen development, prior LT education of neonatal stromal cells was essential because transplant of D3 LTα−/− spleen capsules failed to induce tissue regeneration. This last finding shows clinical relevance to human autotransplantations that use postnatal spleen tissue, and suggests that embryonic spleen development and postnatal spleen development are regulated by different cells or signaling events.

Materials and Methods

Mice

BALB/cEiSlc and C57BL/6JmsSlc mice were purchased from Japan SLC. B6.129S-Rag1tm1Mom (Rag1KO), B6.129P2(Cg)-Ror1tm2Litt/Ror1tm2Litt (Ror1KO), B6.129S2–LacZtm1OriJ (LTα−/−), and B6.129(Cg)-Tg(CAG-Bgeo/GFP) 21Lbe/J (B6.GFP) mice were obtained from The Jackson Laboratory. All mice were housed under specific pathogen–free conditions in our animal facility.

Transplantation and immunization

Spleens were aseptically removed from 3-d-old, 8 d-old, or 8-wk-old mice into 5 ml PBS. Spleens were dissociated between two sterile microscopes and slides and passed through a 100-μm cell strainer (BD Biosciences) to separate suspendable hematopoietic cells from nonsuspendable capsule slides. Supernatant containing suspendable cells was discarded, and nonsuspendable stromal cell–associated capsule tissue was transferred into a 12-well plate containing PBS. Plates were kept on ice until transplantation into the renal subcapsular space of 7-wk-old BALB/c recipients. For primary immunization, 100 μg 4-hydroxy-3-nitrophenylacetate (NP)15–OVA precipitated in alum was injected i.p. for 12-wk-old mice harboring regenereated spleen transplants. For secondary immunization, mice were boosted after 4 wk with 100 μg soluble NP-OVA administered i.v.

Single-cell dissociation

Spleen capsule stromal tissue was enzymatically dissociated into a single-cell solution by incubating tissue in 2 ml supplemented DMEM containing 1 mg/ml Collagenase IV (Invitrogen), 40 μg/ml DNase I (Sigma-Aldrich), and 2% FBS (Life Technologies) for 20 min at 37°C with constant stirring. Incubation was repeated twice more with addition of medium containing 1 mg/ml Collagenase D (Roche), 40 μg/ml DNase I (Sigma-Aldrich), and 2% FBS before filtration through a 100-μm strainer cell. ELISPOT assay

The frequency of NP-specific low- and high-affinity Ab-forming cells (AFCs) from transplants was assessed by ELISPOT assay using NP-BSA–coated filter plates. Hydrophobic polyvinylidene difluoride filters of MultiScreen-IP Filter Plate (Millipore) were coated with 15 μg/ml NP−BSA (for high-affinity AFCs) or NP−pBSA (for low-affinity AFCs) at 4°C overnight, then blocked with RPMI 1640/10% FCS for 30 min at room temperature. Splenocytes (105 cells per well) or cells from transplants (2×106 cells per well) were incubated on the filters in 96-well plates at 37°C, 5% CO2 for 3 h. Wells were washed five times with PBS, then incubated with HRP-conjugated goat anti-mouse IgG, and IgM Abs for 2 h at room temperature. After washing, filters were visualized with AEC (BD Bio-Sciences).

Abs and secondary reagents

FFTC, PE–, Alexa Fluor 647–, eFluor 450–, allophosphocyanin-conjugated or unconjugated anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD16/32 (Fc Block; 2.4G2), anti-CD19 (1D3), anti-CD43 (87), anti-B220 (RA3–6B2), anti–FDC-M1, and anti–MAdCAM-1 (MECA-367) Abs were all purchased from BD Pharmingen. Allophosphocyanin–eFluor 780–, PE–, Alexa Fluor 488–, or biotin-conjugated anti-CD3 (590), anti-CD45 (30-F11), anti-CD45RB (C363.16A), anti-CD105 (M71/18), anti-B220 (RA3–6B2), anti-F4/80 (BM8), anti–gp38 (eBio8.1.1), anti–Gr-1 (RB6–8C5), and anti–LTβR (eBio3C8) Abs were purchased from BioLegend. PE–, Alexa Fluor 488–, or Alexa Fluor 647–conjugated anti–CD90.2 (30–H12), anti–MAdCAM-1 (MECA-367), anti–F4/80 (CI–A3–1), and isotype control Rat IgG1k (RTK2071) Abs were purchased from BioLegend. Unconjugated anti–ER–TR7 and FDC–M2 Abs were purchased from Santa Cruz Biotechnology and ImmunoKontact, respectively. Alexa Fluor 488– or Alexa Fluor 594–conjugated donkey anti–rat secondary reagents were purchased from Molecular Probes. PECy7– and eFluor 660–conjugated streptavidin secondary reagents were purchased from BioLegend and eBioscience, respectively.

Flow cytometry

For discrimination of dead cells, propidium iodide (100 μg/ml) was added to cell suspensions. Flow cytometry acquisition was performed on a FACS Canto II (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

Immunostaining

Lymphoid organs and transplants were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek) and frozen in liquid nitrogen. Cryostat sections, each 7 μm thick, were prepared and placed on glass slides (Matsunami). Sections were stored at –80°C until use. For immunostaining, slides were dried and fixed in acetone for 5 min, followed by three 5-min washes in PBS. After blocking with 1% BSA/0.01% NaN3 in PBS for 30 min at room temperature, sections were incubated for 1 h at room temperature with appropriate Abs or secondary fluorochrome-conjugated reagent diluted in blocking buffer. Each incubation step was followed by three 5-min washes in PBS. Images were captured on an Axio Imager.A2 microscope (Zeiss).

H&E staining

Slides containing tissue sections dried and fixed in acetone were washed three times in PBS. Staining was performed with Mayer’s hematoxylin solution for 3 min, followed by brief rinses in 0.2% HCl solution and H2O. Slides were counterstained with 0.5% eosin Y solution for 3 min, rinsed briefly in H2O, and dehydrated in an ascending series of 90%, 95%, and 99.5% ethanol, before three changes of xylene.

Statistics

The results are expressed as mean ± SEM. All statistical analyses were performed using Prism (GraphPad Software 6c), using an unpaired two-tailed t test. A p value < 0.05 was considered significant.

Results

Spleen tissue regenerates from neonatal spleen capsule

To determine whether spleen tissue can regenerate from neonatal spleen stromal cells, D3 mouse spleen was dissociated to separate stromal cell–associated spleen capsule from hematopoietic cells (Fig. 1A). Spleen capsules were grafted under the kidney capsule of adult recipient mice before analysis after 4 wk. Regenerated spleen tissue could be distinguished from host kidney as morphologically dark red tissue (Fig. 1A), displaying characteristic spleen microarchitecture, including RP and WP, organized T and B compartments, follicular dendritic cells (FDCs), central arterioles (CAs), MZs, conduit structures, and RP macrophages (Fig. 1B).

Donor and host requirements for spleen neogenesis

Grafting of whole-spleen capsules from D3 donors was both highly efficient and reproducible, with 83% (20 of 24) grafts regenerating spleen tissue (Fig. 1C). In addition, 9 of 12 grafts analyzed showed correct T and B cell segregation. Capsule tissue could also be divisible into halves or quarters while retaining regenerative capacity (Supplemental Fig. 1A). However, in contrast to whole- or half-spleen capsule transplants, tissue regeneration from quarter-divided capsules did not result in distinct MZ formation (Supplemental Fig. 1B) and contained significantly lower B cell percentages but equivalent percentages of T cells (Supplemental Fig. 1C). Spleen capsules from D8 mice were also capable of regenerating spleenlike tissue, with 85% of transplanted grafts recovered; however, these tissues did not display correct WP T and B cell compartmentalization (0 of 5 grafts analyzed; Fig. 1C, 1D). Adul spleen capsule grafts also failed to recapitulate normal
FIGURE 1. Neonatal spleen capsule transplantation regenerates complete spleen tissue. (A) Scheme showing spleen capsule transplantation and regeneration of morphologically dark-red spleen tissue above host kidney. (B) Regenerated spleen tissue was collected and analyzed by immunofluorescent section staining to detect formation of spleen tissue structure. Sections were stained with indicated markers identifying lymphoid T and B cell compartments, RP and WP, CAs, FDCs, MZ, and conduit structures. Areas encircled in yellow delineate lymphoid follicles. Original magnification 310. Scale bar, 200 μm. (C) Tissue regeneration efficiency decreases with spleen capsule age, as demonstrated by transplant of D3, D8, and adult (8 wk) capsule grafts and enumeration of gross spleen development and corresponding compartmentalization of T and B cells. (D) Representative immunofluorescence images of tissues regenerated from D3, D8, and adult capsule grafts visualized using Abs against CD90.2 and CD19. Scale bar, 200 μm. Sections are representative of two to four independent experiments per time point, with two or more sections taken from each graft. (E) To determine whether spleen tissue regenerates independently of recipient age, D3 spleen capsules were transplanted into recipient mice aged 6 or 30 wk, and the recovery of regenerated spleen tissue was enumerated after 4 wk. (F) Tissues were analyzed by flow cytometry to determine percent of T and B cells in comparison with

(Figure legend continues)
spleen tissue development (Fig. 1C, 1D). In contrast, an increasing recipient age did not adversely affect tissue regeneration with mice aged 6 and 30 wk, both supporting 92% of spleen capsule grafts (Fig. 1E). Regenerated tissues from either young or old recipients also showed homeostatic T and B cell levels (Fig. 1F), and WP formed discrete lymphoid compartments indicative of normal spleen regeneration (Fig. 1G).

Kinetics of spleen tissue formation

Preparation of neonatal spleen capsules by mechanical dissociation between microscope slides disrupts tissue integrity compared with whole native spleen (Fig. 2A), verifying that spleen regeneration after 4 wk is a result of new tissue synthesis. To visualize spleen regeneration from capsule tissue, grafts were analyzed 2 d, 1 wk, 2 wk, 4 wk, and 9 wk after transplant (Fig. 2B). Separation of F4/80+ RP and WP was evident by 2 d; however, lymphocyte migration into WP areas was not observed. T and B cells interspersed infrequently throughout graft tissue possibly represented donor-derived lymphocytes carried over from capsule preparation. CD11b+ myeloid cells were highly abundant, and although a few CD105+MAdCAM-1+ cells reflecting marginal zone reticular cells (MRCs) were detected, typical MZ arrangement was not evident. Beginning at 1 wk post transplant, an emergence of T cells, B cells, and FDCs was observed in WP areas of grafts. However, only after 2 wk was organization of lymphocytes into distinct T and B cell compartments with MZ boundaries visible. The size of lymphoid follicles continued to increase up to 4 wk post transplant.

Analysis of spleen regeneration by flow cytometry also showed a predominance of myeloid cells at early stages (2 d; 68% ± 4%, mean ± SEM; n = 4), declining to 4.6% ± 0.5% over 9 wk (Fig. 2C). Over the same period, T cells increased 10-fold from 3% ± 0.6–30% ± 4.5% and B cells from 19% ± 2–53% ± 4.5%. At 4 wk post transplant, lymphoid cell populations present in regenerated spleens were similar to those in native adult spleen, showing an approximate 1:1.5 T cell/B cell ratio (Fig. 2D). In contrast, myeloid cells showed a heavy bias toward granulocytes, indicating that the myeloid cell compartment had not reached steady-state levels, even after 4 wk post transplant. Lymphocytes present in regenerated spleens were host derived, as shown by wild-type spleen capsule transplants into B6.GFP recipients, leading to >93% GFP+ host-derived T and B cells present in grafts after 1 wk, and 99% GFP+ after 2 wk (Fig. 2E).

Induction of secondary Ab responses

To determine the capacity of regenerated spleen to support immune responses, Ab production following antigenic immunization was assessed by ELISPOT assay. Mice previously transplanted with neonatal spleen capsule grafts (and thus harboring regenerated tissues) were immunized with NP-OVA/Alum and analyzed after 2 wk for generation of primary immune responses (Fig. 3A). Immunized mice were also retained for secondary immune response analysis by Ag boosting 4 wk after initial priming and analysis of tissues 1 wk later. Regenerated spleen tissue was capable of generating Ag-specific low-affinity IgM Ab after 2 wk at levels higher than those of native immunized spleen (Fig. 3B). Consistent with primary immune responses, neither immunized native or regenerated spleens induced high-affinity IgM or IgG1 Ab production. After secondary boosting with NP-OVA, Ag-specific high-affinity IgM and both low- and high-affinity IgG1 Ab-forming cells were detected in significantly higher numbers in regenerated spleens compared with nonimmunized spleen (Fig. 3C), demonstrating the capacity for regenerated tissue to sustain Ab class switching and affinity maturation in a secondary immune response.

**Graft-derived lymphocytes are dispensable for spleen development**

Mechanical separation of capsule tissue from neonatal spleen leads to enrichment of stromal cells (16). However, analysis of spleen capsule stromal preparations revealed residual hematopoietic T and B cells (data not shown). To exclude the contribution of donor-derived lymphocytes to spleen regeneration, spleen capsules from Rag1KO donors were transplanted into syngenic hosts. Development of spleen tissue structure from Rag1KO capsules after 4 wk was comparable to control wild-type grafts, with 15 of 16 grafts showing T and B cell zone arrangement, FDCs, and MZs (Fig. 4A). Percentages of lymphocytes and myeloid cells in regenerated spleen were also similar between transplants (Fig. 4B), confirming that tissue regeneration is donor-lymphocyte independent, and that neonatal spleen stromal cells are capable of inducing spleen tissue neogenesis.

**Gross spleen tissue regeneration requires LT education of stromal cells**

LTi cells and LT signaling are essential for embryonic LN development, although both are dispensable for gross spleen tissue formation (11, 13, 17–19). To determine whether LTi are present in neonatal spleen capsule preparations, freshly isolated D3 splenocytes or enzymatically digested capsule tissues were analyzed by flow cytometry, revealing the presence of a CD45+CD3− CD19− CD11b+CD4+IL-7Rα+ LTi population (Fig. 5A). To assess whether LTi present in grafts were necessary for tissue regeneration, spleen capsules from 3-d-old RORγt−/− donors were transplanted into adult wild-type recipients (Fig. 5B). Similarly, the graft requirements for LT exposure during postnatal development were assessed by transplanting D3 donor LTα−/− spleen capsules. In comparison with spleens regenerated from WT neonatal spleen capsule grafts (14 of 19 grafts transplanted), D3 LTα−/− spleen capsules showed no capacity for spleen development (0 of 13 grafts transplanted). In contrast, 5 of 7 RORγt−/− spleen capsule grafts regenerated tissue displaying abnormal WP organization and a general lack of MZ structure (Fig. 5B, third column). Therefore, donor-derived LTi retained in spleen capsule grafts are dispensable for initiating gross tissue regeneration but are required for WP organization. In contrast, early developmental LT signaling in spleen, prior to stromal capsule isolation and grafting, appears essential for subsequent tissue regeneration.

In the reverse transplantation, wild-type D3 spleen capsules were transplanted into RORγt−/− or LTα−/− recipients to assess host requirements for LTi and LT signaling (Fig. 5C). In RORγt−/− mice lacking LTi, wild-type spleen capsules were capable of spleen tissue development similar to native RORγt−/− spleen structure. Formation of WP was evident in tissue regenerated in RORγt−/− hosts; however, T and B cell compartments lacked clear segregation compared with native adult spleen. The presence of FDCs and diffuse MZs was, though, detected. LT-deficient LTα−/− hosts also supported the formation of spleen tissue from WT neonatal spleen capsules. Similar to native LTα−/− spleen,
Regenerated tissues showed poor formation of WP with no lymphocyte segregation, FDCs, or MZ structures. Therefore, regeneration of spleen tissue in either LT- or LTi-deficient hosts mimics their respective native mutant spleen phenotypes, supporting an argument that spleen capsule transplants reflect a functional model for spleen development. Collectively, the failure...
of LTα−/− D3 spleen capsules to develop tissue in WT hosts (Fig. 5B), combined with observations that WT D3 spleen tissue regenerates independently of adult LTi and LT (Fig. 5C), suggests that spleen stroma is initially primed by LT signaling during neonatal development, and this is sufficient to sustain gross spleen tissue formation with further LTi/LT engagement mediating correct organization of WP.

Analysis of stromal populations in neonatal and adult spleen capsule

To determine stromal changes during spleen postnatal ontogeny, five stromal CD45− cell populations were identified flow cytometrically as fibroblastic reticular cells (gp38+CD31+), CA (gp38−CD31+), RP sinusoids (gp38−CD105+), RP stroma (gp38−F4/80+), and MRCs (gp38−MAdCAM-1hi), with spleen capsules analyzed at 3 d, 8 d, and 8 wk of age (Fig. 6A). Between 3 and 8 d, all splenic stromal populations showed an increase in relative percentage, with the exception of MAdCAM-1 hi cells, which decreased significantly (Fig. 6B, upper panel). Moreover, the absolute numbers of MAdCAM-1 hi cells declined more than 2-fold between 3 and 8 d (Fig. 6B, lower panel), despite an overall stability in total stromal cell numbers (data not shown). In contrast, over the same time period, CD31+, CD105+, F4/80+, and gp38+ stromal populations increased in absolute cell number. The capacity for D3 spleen capsule grafts to completely regenerate tissue, which diminishes by 8 d of age, correlates with the availability of MAdCAM-1 hi cells, which also declines rapidly during early neonatal development. MAdCAM-1 hi cells also express LTβR (Fig. 6C), in line with the capacity to respond to LT signaling. The median fluorescence intensity of surface LTβR on MAdCAM-1 hi cells was comparable to that on gp38+ cells but significantly higher than that on CD31+ spleen stromal cells (Fig. 6D). Bimodal LTβR expression on CD105+ cells revealed that only CD105 hi, not CD105 lo cells, expressed LTβR.

Discussion

Spleen regeneration has previously been demonstrated from whole embryonic spleen in mice (14, 15) and dissociated neonatal splenic units in rats (20), whereas in the clinical setting human spleen autotransplantations involve spleen fragmentation into slices and retransplant into the patient (4). We now demonstrate that in contrast to bulk tissue transplants, neonatal spleen capsule alone or in fragments thereof are sufficient to regenerate spleen tissue in the murine mouse model (Fig. 2A, Supplemental Fig. 1). This finding identifies an important role for spleen stromal cells in tissue development, supported by the transplant and regeneration of Rag1KO spleen capsules, which lack graft-derived lymphocytes. Transplant of spleen capsule alone also likely avoids mass necrosis of spleen slices observed in murine transplantation models (6). We now demonstrate that in contrast to bulk tissue transplants, neonatal spleen capsule alone or in fragments thereof are sufficient to regenerate spleen tissue in the murine mouse model (Fig. 2A, Supplemental Fig. 1). This finding identifies an important role for spleen stromal cells in tissue development, supported by the transplant and regeneration of Rag1KO spleen capsules, which lack graft-derived lymphocytes. Transplant of spleen capsule alone also likely avoids mass necrosis of spleen slices observed in murine transplantation models (6).
display immune function sustaining Ag-specific Ab responses, including affinity maturation and Ig-class switching, strengthening the idea that spleen capsule transplants represent a viable option for spleen regeneration.

Murine 3-d-old donor spleen capsules are capable of full tissue regeneration. However, by 8 d, the capacity for normal WP development becomes restricted, a result in line with spleen auto-transplantation studies performed in rat models (21). Because the immune function of spleen closely correlates with correct WP organization (21, 22), development of WP compartments represents a principal objective for spleen regenerative therapies. An understanding of the mechanisms underlying neonatal spleen tissue regeneration and the stromal cells responsible for both RP and WP formation could therefore greatly enhance spleen transplant efficiency, especially from older donors.

LT is a key molecule for LN organogenesis and remodeling (7, 23). For spleen development, the role of LT has been studied in experiments antagonizing LTα1β2 signaling during spleen organogenesis. A time window at which LT exerts the highest influence over WP formation was defined, with treatment of mice with LTα2R-immunoglobulin at 0.5 d, but not at 7 d, leading to defects in adult T cell zone development (24). These observations indicate that for WP formation, LT signaling is most important during early postnatal spleen development. Moreover, either LTi or B cells appear capable of delivering LT signaling during this period. In Rag1KO mice lacking lymphocytes but not LTi, reconstitution by LTα2/2 splenocytes establishes proper WP formation (25), whereas normal WP is also displayed in LTα2/2 mice carrying a B cell-specific LTα transgene (24).

In contrast, the roles for LTi and LT signaling in spleen tissue organogenesis are less clear. Transplant of E15 LTα2/2 spleen into WT hosts leads to generation of full spleen tissue (14), suggesting LTα1β2 is not required during early embryogenesis. Our data show that this regeneration capacity ceases by transplant of D3 postnatal LTα2/2 spleen capsules, indicating that a LTα1β2 requirement exists between E15 and D3 post birth. In contrast, D3 RORγt−/− spleen transplants are capable of tissue regeneration, suggesting that LT signaling to spleen capsule stromal cells is not delivered by LTi. Therefore, in addition to the role of LT in adult WP formation, we propose a second function for LT signaling in neonatal spleen tissue organogenesis. However, this contrasts with long-held observations that spleen arises during embryonic de-

**FIGURE 4.** Neonatal spleen stromal cells regenerate tissue independently of graft-derived lymphocytes. To determine whether lymphocytes present in neonatal spleen capsules were required for spleen development, D3 spleen capsule (CAP) grafts from Rag1KO mice lacking lymphocytes were transplanted into syngeneic BALB/c recipients. Wild-type (WT) BALBc spleen capsule grafts were transplanted as a positive control. (A) Tissues were collected after 4 wk and assessed by immunofluorescent microscopy to determine formation of spleen structure. Original magnification ×10. Scale bar, 200 μm. (B) Percent of lymphoid, myeloid, and dendritic cells in regenerated tissues was assessed by flow cytometry. n = 3–4. Error bars indicate SEM.
development through to adulthood in LTα1β2-deficient mice (11), raising the possibility that embryonic and neonatal spleen developmental pathways are separately regulated.

During embryogenesis, spleen is believed to develop from a mesoderm-derived cell layer, the splanchnic mesodermal plate (26). Splenic stroma, including fibroblastic reticular cells, FDCs, and pericytes, develops directly from embryonic mesenchymal cells (27). Therefore, in LT-deficient mice exhibiting spleen formation, these mesenchymal-derived cell lineages must be capable of driving spleen organogenesis from embryonic through to adult-stage tissue independently of LT signaling, consistent with the presence of spleen in LT-deficient mice (11). In this situation, LT-dependent endothelial organizers in conjunction with LTi may mediate correct adult WP formation (24). In contrast, spleen regeneration from neonatal spleen capsule transplants relies upon LTα1β2-educated endothelial organizers, and in the absence of LT signaling shortly after birth, capsules fail to regenerate spleen tissue. Although this model accounts for a lack of tissue development observed from

**FIGURE 5.** Donor and host LTi cell and LT requirements for neonatal spleen tissue regeneration. (A) The presence of donor-derived CD45+ CD3−CD19−CD11b+CD4+CD127 (IL-7R)− LTi in spleen capsule grafts was investigated using flow cytometry. D3 splenocytes were assessed in parallel as a positive control. Data are representative of two independent experiments. (B) Preactivation of neonatal spleen capsule grafts by LT signaling, but not the presence of donor-derived LTi, is required for tissue formation. D3 LTα−/− (LT α-deficient) or RORγt−/− (inducer cell-deficient) spleen capsule (CAP) grafts were transplanted into adult wild-type C57BL/6 (B6) hosts. Wild-type B6 D3 capsules were transplanted as positive controls, and regenerated tissues were analyzed after 4 wk by immunofluorescence microscopy. Native D3 RORγt−/− or LTα−/− spleen is shown for comparison. Original magnification ×10. Scale bar, 200 μm. (C) Host-derived LTi cells or LT expression is dispensable for neonatal spleen regeneration. D3 spleen CAP grafts from wild-type B6 donors were transplanted into RORγt−/− or LTα−/− recipients before analysis at 4 wk. Native adult spleen from C57BL/6, RORγt−/−, or LTα−/− mice is shown for comparison. Insets, Enlarged areas of T and B cell interface.
LT-deficient neonatal spleen capsule grafts, the proposal of an endothelial-lineage spleen organizer departs from the classical paradigm that lymphoid organizers are mesenchymally derived (32). Validation of such a model therefore requires formal identification of an endothelial spleen organizer cell population with demonstration of tissue-organizing ability.

Disclosures
The authors have no financial conflicts of interest.

References


### Supplementary Figure 1

#### A

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<th>Spleen tissue regeneration (%)</th>
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<td>1 CAP</td>
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<td>34/36 (94%)</td>
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#### B

CD90.2  CD19  F4/80  CD45RB  MAAdCAM-1  B220  B220  FDC-M2  H&E

#### C

T cells

- % CD90.2
- % CD19

B cells

- % CD90.2
- % CD19
Supplemental Figures

SUPPLEMENTAL FIGURE 1. Division of neonatal spleen capsules into smaller fractions does not inhibit tissue regeneration. (A) 3-day old spleen capsules were isolated and transplanted whole (1 CAP), in halves (0.5 CAP) or in quarters (0.25 CAP) into adult recipient mice with the efficiency of spleen tissue regeneration determined after 4 weeks transplantation. (B) Tissues were analysed for spleen tissue structure under immunofluorescence (Original magnification x10. Scale bar: 200µm) and hematoxylin and eosin (H&E; 4x, Scale bar: 500µm) staining identifying T and B cell compartments, red pulp (RP), white pulp (WP), marginal zones (MZ) and follicular dendritic cells (FDC). Areas encircled in yellow delineate lymphoid follicles. (C) Flow cytometry analysis of grafts showing percent T and B cells compared with control spleen. Each point on the graph represents one tissue with horizontal bars representing the mean. **, P<0.01; unpaired two-tailed t test.