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*J Immunol* published online 20 June 2014
http://www.jimmunol.org/content/early/2014/06/19/jimmunol.1302115

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http://www.jimmunol.org/content/suppl/2014/06/19/jimmunol.1302115.DCSupplemental

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

Jonathan K. H. Tan 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: 000–000.

Spleen tissue autotransplantations are used as a means of conserving spleen function in patients undergoing splenectomy. As an organ with immune function, spleen contains multiple cell populations capable of responding to both innate and acquired arms of immunity. Innate immune cells positioned in the marginal zone (MZ) of spleen react quickly to blood-borne bacteria and viruses, clearing pathogens via macrophage-mediated phagocytosis and Ab release into the bloodstream following B cell activation (1). Without a rapid innate immune response, asplenic patients face a risk of overwhelming postsplenectomy infection, especially from encapsulated bacteria such as Streptococcus pneumoniae (2). Although the risk of overwhelming postsplenectomy infection is relatively low, progression to fulminant sepsis can occur rapidly and mortality rates are estimated to reach 50–70% (2, 3). To retain spleen function following spleenectomy, a method for whole-spleen tissue fragmentation into thin slices and reimplantation into omental pouches has been studied in animal models and applied to human surgical procedures (4, 5).

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Received for publication August 9, 2013. Accepted for publication May 25, 2014.

This work was supported by Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan, 19059015 (to T.W.) and a Japan Society for the Promotion of Science Postdoctoral Fellowship for Foreign Researchers (to J.K.H.T.).

J.K.H.T. initiated the study, designed and performed experiments, analyzed data, and wrote the manuscript; and T.W. designed and supervised research and edited the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AFC, Ab-forming cell; CA, central arteriole; D3, day 3; D8, day 8; E15, embryonic day 15; FDC, follicular dendritic cell; LN, lymph node; LT, lymphotoxin; LTi, lymphoid tissue inducer; LTo, lymphoid tissue organizer; LTβR, LT-β receptor; MRC, marginal zone reticular cell; MZ, marginal zone; NP, 4-hydroxy-3-nitrophenylacetyl; RP, red pulp; WP, white pulp.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302115
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/cCrSlc and C57BL/6Jmslce mice were purchased from Japan SLC. B6.129S-Rag1tm1Mom (Rag1KO), B6.129P2(Cg)-Rorc−/−(RORγt−/−), B6.129S2-Lat−/−(LTA−/−), and B6.129(Cg)-Tg(CAG-Bgeo/GFP) (21hobo) (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 microscope slides and passed through a 100-μm cell strainer (BD Biosciences) to separate suspended hematopoietic cells from nonsuspended capsule tissue. Supernatant containing suspended cells was discarded, and nonsuspended 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-nitrophenylacetyl (NP) 15–21Lbe/J (B6.GFP) mice were obtained from the Jackson Laboratory. All mice were housed under specific pathogen-free conditions in our animal facility.

**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 cell strainer.

**ELISPOT assay**

The frequency of NP-specific low- and high-affinity Ab-forming cells (AFCs) from transplants was assayed 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 3–15/BSA (for low-affinity AFCs) at 4°C overnight, then blocked with RPMI 1640/10% FCS for 30 min at room temperature. Splenocytes (1×106 cells per well) or cells from transplants (2.5×106 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 anti-mouse IgG1 and IgM Abs for 2 h at room temperature. After washing, filters were visualized with AEC (BD Biosciences).

**Abs and secondary reagents**

**FTTC–PE**, Alexa Fluor 647–eFluor 450–, allophtocyanin-conjugated or unconjugated anti-CD11b (M1/70), anti-CD11c (IHL3), anti-CD16/32 (Fc Block: 2.4G2), anti-CD19 (1D3), anti-CD43 (S7), anti-B220 (RA3-6B2), anti–FDC-M1, and anti–MaDcAM-1 (MECA-367) Abs were all purchased from BD Pharmingen. Allophtocyanin–eFluor 780–, PE, Alexa Fluor 488–, or biotin-conjugated anti-CD31 (390), anti-CD45 (30-F11), anti-CD45R (C363.16A), anti-CD110 (M7/18), and anti-B220 (RA3-682), 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 (Cl:4A3-1), and isotype control Rat IgG1k (RTK207l) Abs were purchased from BioLegend. Unconjugated anti–ER–TR7 and FDC–M2 Abs were purchased from Santa Cruz Bio-technology 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 with 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 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). Adult 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 ×10. 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 capsules (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-7R+ 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 capsule spleens. 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

![Regenerated Tissues](image)

**FIGURE 2.** Regeneration kinetics of spleen tissue from neonatal capsule grafts. (A) Neonatal spleen capsule preparations lack intact lymphoid compartments or stromal networks. Native spleen from D3 mice, or capsules prepared by mechanical dissociation of spleen, were sectioned and stained with CD90.2, CD19, ER-TR7, or CD31 for immunofluorescence analysis. Original magnification ×10. Scale bar, 200 μm. Arrows indicate CAs. FRC, fibroblastic reticular cells. Insets, Enlarged areas showing CAs. (B) D3 spleen capsules transplanted into adult recipients were removed after 2 d, 1, 2, and 4 wk to assess the developmental kinetics of spleen regeneration, using immunofluorescence staining with indicated markers. Arrows indicate FDCs. Original magnification ×10. Scale bar, 200 μm. (C) Regeneration kinetics of lymphoid and myeloid cell populations in grafts out to 63 d. CD90.2+ T cells, B220+ B cells, CD11b+ myeloid cells, and CD11c+ dendritic cells. Values are mean ± SEM, n = 4. (D) Graph of myeloid and lymphoid cell distribution in regenerated spleen after 4 wk (4w) compared with native adult control spleen. Cell populations were identified flow cytometrically as CD19+ B cells, CD90.2+ T cells, CD11c+ dendritic cells (DC), Gr-1+ granulocytes, CD11b+CD43 inflammatory monocytes, and CD11b+CD43 resident monocytes. (E) To determine the host versus donor cell contributions of regenerated spleen tissue, C57BL/6 (B6) spleen capsules were transplanted into C57BL/6.GFP (B6.GFP) recipients, and host (GFP+) versus donor (GFP−) CD19+CD90.2+ lymphocytes were assessed after 1 and 2 wk by flow cytometry (n = 3; error bars indicate SEM). Native spleen from wild-type C57BL/6 and C57BL/6.GFP mice was used as a control.
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-1hi cells, which decreased significantly (Fig. 6B, upper panel). Moreover, the absolute numbers of MAdCAM-1hi 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-1hi cells, which also declines rapidly during early neonatal development. MAdCAM-1hi 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-1hi 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 CD105hi, not CD105lo 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). Moreover, regeneration occurs quickly, with host-derived lymphocytes repopulating grafts within 1 wk post transplant, and grafts persist within hosts without reabsorption into the body (data not shown). Regenerated spleens also

FIGURE 3. Functional capacity of regenerated spleen tissue. The capacity for regenerated spleen grafts to function as immune tissue was assessed by ELISPOT assay for Ag-specific Ab formation and class switching after primary and secondary immune responses. (A) Procedure for analysis of Ab formation in grafts. D3 spleen capsules were transplanted into nonsplenectomized adult recipients, and 5 wk after spleen tissue regeneration, mice were immunized by i.p. injection of NP-OVA/alum Ag. After 2 wk, grafts and native spleens were collected and analyzed by ELISPOT to detect Ag-specific AFCs produced in a primary immune response. For secondary immune response analysis, mice were boosted 4 wk after initial priming by i.v. NP-OVA administration and tissues collected one week later for ELISPOT analysis. (B) Frequency of NP-specific IgM and class-switched IgG1 AFCs after 2 wk primary immune responses in naive spleen (○), immunized spleen (■), and immunized grafts (▲). Low-affinity IgM or IgG1 Ab formation was assessed by binding to NP30-coated ELISPOT plates, whereas NP3 was used to assess high-affinity Ab production. (C) Analysis of secondary immune responses after 5 wk primary immunization and Ag boosting. Data are representative of three independent experiments (two to five mice per group). *p < 0.05, ***p < 0.001.
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 LTR1β2 signaling during spleen organogenesis. A time window at which LT exerts the highest influence over WP formation was defined, with treatment of mice with LTR1β2 immunoglobulin at 0.5 d, but not at 7 d, leading to defects in adult T cell zone development (24). This observation indicates an early, not late, postnatal requirement for LT. However, before birth LTR1β2 signaling has less importance because antagonism of LTR1β2 binding at embryonic day 14 or embryonic day 17 has comparatively fewer effects on adult WP segregation (24).

Recruitment of LTi and clustering around LTo-like organizer cells during embryonic spleen development also occurs independently of LTR1β2 signaling (25). Only during early postnatal spleen development is cell surface expression of LTR1β2 increased on LTi and B cells, with spleen development up to 3-4 d similar between LTRα−/− and wildtype mice (15). Overall, these factors 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 LTRα−/− splenocytes establishes proper WP formation (25), whereas normal WP is also displayed in LTRα−/− mice carrying a B cell-specific LTRα transgene (24).

In contrast, the roles for LTi and LT signaling in spleen tissue organogenesis are less clear. Transplant of E15 LTRα−/− spleen into WT hosts leads to generation of full spleen tissue (14), suggesting LTR1β2 is not required during early embryogenesis. Our data show that this regeneration capacity ceases by transplant of D3 postnatal LTRα−/− spleen capsules, indicating that a LTR1β2 requirement exists between E15 and D3 post birth. In contrast, D3 RORγ−/− 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-
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 lineage organizer cells can develop from the splanchnic mesodermal plate.

To account for the inability of spleen to regenerate from LT-deficient neonatal capsule grafts versus the presence of spleen in LTα1β2 mice, we propose a model whereby nonmesenchymal LTα1β2-educated endothelial lineage organizer cells can develop from the splanchnic mesodermal plate. Such organizer-like cells expressing CD31 would not arise from embryonic mesenchymal cells (27), suggesting the existence of a second endothelial-lineage spleen organizer population. Indeed, LN CD31+ endothelial cells have now been recognized as a critical component for tissue formation in an LTβR-dependent manner (29). Early mesenchymoangioblasts derived from human embryonic stem cell–induced mesenchymal precursor cells have been shown to produce both lineages of mesenchymal stem cells and endothelial cells (30, 31), so supporting a hypothesis that both mesenchymal and endothelial lineage organizer cells can develop from the splanchnic mesodermal plate.
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

FIGURE 6. Spleen stromal cell analysis at neonatal and adult stages. (A) Spleen capsules were isolated postnatally at 3 d, 8 d, and 8 wk of age, and stromal markers assessed by flow cytometry. Representative plots of 3-d-old spleen capsule are shown. Numbers in quadrants indicate percent of cells, and elliptical gates define MadCAM-1hi-expressing cells. (B) The percentages of gp38+, gp38−CD31+ (CD31+), gp38−CD105+ (CD105+), gp38−F4/80+ (F4/80+), and gp38−MadCAM-1hi (MadCAM-1hi) stromal populations among CD45+ cells were analyzed (upper panel) and absolute cell numbers determined (lower panel). Error bars indicate SEM. *p < 0.05. Data are representative of four to six independent experiments per time point. (C) Surface LTβR expression on 3-d spleen stromal subsets was determined by flow cytometry (solid lines). Dotted histograms represent cells stained with isotype-matched control Abs. Data are representative of three independent experiments. (D) Median fluorescence intensity of LTβR expression after subtracting isotype background fluorescence. Data represent mean values and SEM from three independent experiments. *p < 0.05, ***p < 0.001.


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### C

![Graph showing T cell and B cell percentages](image)

**Supplementary Figure 1**
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.