The Requirement of Membrane Lymphotoxin for the Presence of Dendritic Cells in Lymphoid Tissues

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Summary

Although several cytokines, including tumor necrosis factor (TNF), can promote the growth of dendritic cells (DCs) in vitro, the cytokines that naturally regulate DC development and function in vivo have not been well defined. Here, we report that membrane lymphotoxin (LT), instead of TNF, regulates the migration of DCs in the spleen. $LT\alpha^{-/-}$ mice, lacking membrane $LT\alpha/\beta$ and $LT\alpha_3$, show markedly reduced numbers of DCs in the spleen. Unlike wild-type mice and $TNF^{-/-}$ mice that have densely clustered DCs in the T cell zone and around the marginal zone, splenic DCs in $LT\alpha^{-/-}$ mice are randomly distributed. The reduced number of DCs in lymphoid tissues of $LT\alpha^{-/-}$ mice is associated with an increased number of DCs in nonlymphoid tissues. The number of splenic DCs in $LT\alpha^{-/-}$ mice is restored when additional LT-expressing cells are provided. Blocking membrane $LT\alpha/\beta$ in wild-type mice markedly diminishes the accumulation of DCs in lymphoid tissues. These data suggest that membrane LT is an essential ligand for the presence of DCs in the spleen. Mice deficient in TNF receptor, which is the receptor for both soluble $LT\alpha_3$ and $TNF-\alpha_3$ trimers, have normal numbers of DCs. However, $LT\beta R^{-/-}$ mice show reduced numbers of DCs, similar to the mice lacking membrane $LT\ \alpha/\beta$. Taken together, these results support the notion that the signaling via $LT\beta R$ by membrane $LT\alpha/\beta$ is required for the presence of DCs in lymphoid tissues.

Key words: membrane lymphotoxin • tumor necrosis factor • dendritic cells • lymphotoxin receptor • migration

Soluble lymphotoxin (LT)α[1] and TNF-α are structurally related homotrimers ($LT\alpha_3$ and $TNF-\alpha_3$) that show similar biological activities by binding to either of the two defined TNF receptors, TNFR-I and TNFR-II, leading to activation of a wide variety of inflammatory and immune responses (1, 2). $LT\alpha$ also exists as a membrane ligand by binding to $LT\beta$ to form a membrane $LT\alpha_1\beta_2$ heterotrimer (membrane LT), which shows a high-affinity interaction with $LT\beta$ receptor ($LT\beta R$) but only very low affinity for TNFR-I or TNFR-II. The expression of membrane $LT\alpha_1\beta_2$ is detected on activated T, B, and NK cells, whereas its receptor is expressed exclusively in nonlymphoid tissues (1, 3–5). The role of membrane LT and $LT\beta R$ has been recently revealed by gene targeting. $LT\alpha^{-/-}$, $LT\beta^{-/-}$, and $LT\beta R^{-/-}$ mice all manifest profoundly defective LN and Peyer's patch development and altered splenic structure and B cell follicles (6–9). Blocking membrane LT function during mouse ontogeny by injection of a soluble $LT\beta R$–Ig fusion protein or an anti-$LT\beta$ mAb to pregnant wild-type (wt) mice resulted in the absence of peripheral lymphoid organogenesis in their progeny. Conversely, activation of $LT\beta R$ with an agonistic mAb could restore LN formation in the $LT\alpha^{-/-}$ mice (10, 11). The data prove that signaling via $LT\beta R$ by membrane LT on nonlymphocytes is required for lymphoorganogenesis and the formation of the lymphoid tissue microenvironment.

The formation of microenvironment, such as B cell follicles and T/B cell segregation in lymphoid tissue, may depend on the expression of membrane LT on B cells (12–15). LT may also regulate the localization of various lymphoid and nonlymphoid cells by regulating a series of chemokines in the lymphoid organs. For example, some chemokines produced by stromal cells in B cell follicles direct the polarization of the B cell follicles (16). Although the cell types producing chemokines induced by LT in lymphoid tissue

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[1]*Abbreviations used in this paper:* BM, bone marrow; DCs, dendritic cells; FDCs, follicular dendritic cells; LT, lymphotoxin; MLR, mixed leukocyte reaction; MZs, marginal zones; wt, wild-type.

have not been identified, the expression pattern of chemokines in lymphoid tissues resembles the distribution pattern of follicular dendritic cells (FDCs) in B cell follicles and lymphoid dendritic cells (DCs) in T cell zones (2, 16). Although the role of membrane LT in the regulation of B cell–related events and the maintenance of FDCs is well defined, participation of this regulatory system in DC/T cell events remains unclear. Interestingly, inhibition of the membrane LT pathway has profound effects on several T cell–based disease models, e.g., colitis (17), collagen-induced arthritis (Browning, J.L., and R.A. Fava, unpublished observations), and induction of experimental autoimmune encephalitis (Browning, J.L., and C.L. Nickerson-Nutter, unpublished observation). T lymphocytes are important mediators of immunity, but their function is tightly regulated by DCs (18, 19). One explanation for these observations would be parallel regulation of DC/T cells, similar to that of FDC/B cells, in an LT-dependent fashion (12–16).

Cytokines, such as GM-CSF and TNF, promote the growth of DCs in vitro, but less is known about the regulation of DC distribution and development in vivo (20, 21). Injection of a pharmacological dose of polyethylene glycol–modified GM-CSF into mice only expands the myeloid-related DC subset (22). Interestingly, GM-CSF$^{-/-}$ or GM-CSFR$^{-/-}$ mice show no significant impairment in the development of splenic DCs, suggesting that this cytokine is not absolutely required for DC development (23). Here, we report that LT$\alpha^{-/-}$ or LTβR$^{-/-}$ mice show markedly reduced numbers of splenic DCs but increased numbers of DCs in nonlymphoid tissues. DCs are present in normal numbers and distribution in TNF$^{-/-}$ and TNFR$^{-/-}$ mice. Reconstitution of LT$\alpha^{-/-}$ mice with LT-expressing cells restores the number of DCs in the spleen. On the other hand, removal of LT-expressing cells or blocking membrane LT in wt mice created an impaired DC migration phenotype similar to that seen in LT$\alpha^{-/-}$ mice. These findings strongly suggest that signaling via LTβR by membrane LT is critical for the migration of DCs into lymphoid tissues.

Materials and Methods

Animals. LT$\alpha^{-/-}$ mice (backcrossed to C57BL/6 mice for seven generations) and their wt littermates on a C57BL/6 background were bred under specific pathogen-free conditions as described (6). LTβR$^{-/-}$ mice were provided by Dr. Klaus Pfeffer (Technical University of Munich, Germany) (8). TCR$^{-/-}$, BCR$^{-/-}$, RAG-1$^{-/-}$, TNFR-I$^{-/-}$, and TNF$^{-/-}$ mice as well as CD3ϵ-transgenic mice were purchased from The Jackson Laboratory. B6-Ly5.1 mice were purchased from Frederick Cancer Center, National Cancer Institute, Bethesda, Maryland. Animal care and use were in accordance with institutional guidelines.

Cell Preparation and Staining. Splenic DCs were treated and collected basically according to the method developed by Inaba et al. (24). In brief, spleen fragments were digested with 2 mg/ml of collagenase and 100 μg/ml DNase for 30 min at 37°C and then gently pipetted in the presence of 0.01 M EDTA for 1 min. Single-cell suspensions were stained and analyzed by two-color flow cytometry on a FACScan™ (Becton Dickinson). Biotiny-

lated anti-CD11c and CD11b (Mac-1), FITC-conjugated anti–I-Ab, anti-CD11c, and anti-CD8α antibody were all obtained from PharMingen.

Immunohistology. Spleens were harvested, embedded in OCT compound (Miles-Yeda, Inc.), and frozen at −70°C. Frozen sections (6–10 μm thick) were fixed in cold acetone. Endogenous peroxidase was quenched with 0.2% H$_2$O$_2$ in methanol. After washing in PBS, the sections were stained by first incubating with FITC-conjugated anti-B220 for B cells and biotinylated anti-CD11c for DCs (PharMingen) at 1:50–100 dilution. Horseradish peroxidase–conjugated rabbit anti–FITC (DAKO Corp.) and alkaline phosphatase–conjugated streptavidin (Vector Labs., Inc.) were added 1 h later. Color development for alkaline phosphatase and horseradish peroxidase was performed with an alkaline phosphatase reaction kit (Vector Labs., Inc.) and with 3,3′-diaminobenzidine (Sigma Chemical Co.).

Generation of Reagents that Block Membrane LT Activity. Anti-LTβ antibody and some aspects of the control LTβR–Ig fusion protein used in this study have been previously described (4). The method for the generation of LTβR–Ig fusion protein was used as previously described with a minor modification (4). In brief, cDNA encoding the extracellular domain of murine LTβR was isolated by RT-PCR using the sense primer (5′-AAAGGC-CGCCATGGGCCT-3′) and the antisense primer (5′-TTAA-GCTTCAGTAGCATTGCTCCTGGCT-3′) from mouse lung mRNA, digested by NcoI/HindIII, and then fused to an IL-3 leader sequence in p30242 vector. The fusion fragment was then subcloned into pX58 vector containing the IE-175 promoter and the Fc portion of human IgG1, which was then transfected into BHK/VP16 cells. The mouse LTβR–human Ig in culture supernatants was purified on a protein A column. No difference can be found between LTβR–human Ig in this preparation and a previous LTβR–Ig preparation in Chinese hamster ovary cells (4). To block membrane LT activity in mice, the LTβR–Ig or anti-LTβ antibody (50–100 μg/injection) was given intraperitoneally, and the number of DCs was determined 10–14 d later by either flow cytometry or immunohistology.

Cell Transfer. Bone marrow (BM) cell or splenocyte transfer was performed as previously described (12). In brief, BM-derived DCs (BMDCs) from Ly5.1 mice were obtained by culturing BM cells with GM-CSF (5 ng/ml) and IL-4 (2 ng/ml) according to the procedure developed by Inaba et al. (25). BMDCs (5 × 10⁶) or splenocytes (5 × 10⁷) were intravenously transferred into sublethally irradiated recipient mice (600 rads). Spleens and LN cells were collected for analysis within 24 h after transfer.

Mixed Lymphocyte Reaction. As stimulating cells, splenocytes from wt or LT$\alpha^{-/-}$ mice were isolated by gentle pressure through a cell strainer (Becton Dickinson), or spleen fragments were treated with collagenase as described earlier (24). The stimulating cells were irradiated at 2,000 rads. The LN cells from BALB/c mice were collected by gentle pressure using a cell strainer and cultured in a petri dish for 2 h. The nonadherent LN cells were then harvested and used as the source of responding cells. The different amounts of stimulating cells as indicated and 4 × 10⁵ responding cells were cocultured for 72 h, and [³H]TdR at 1 μCi/ml was added during the last 18 h.

Results and Discussion

Markedly Reduced Numbers of DCs in LT$\alpha^{-/-}$ Mice but Not in TNF$^{-/-}$ Mice. TNF can promote the growth of DCs in vitro (15, 16). To assess the role of TNF in the de-
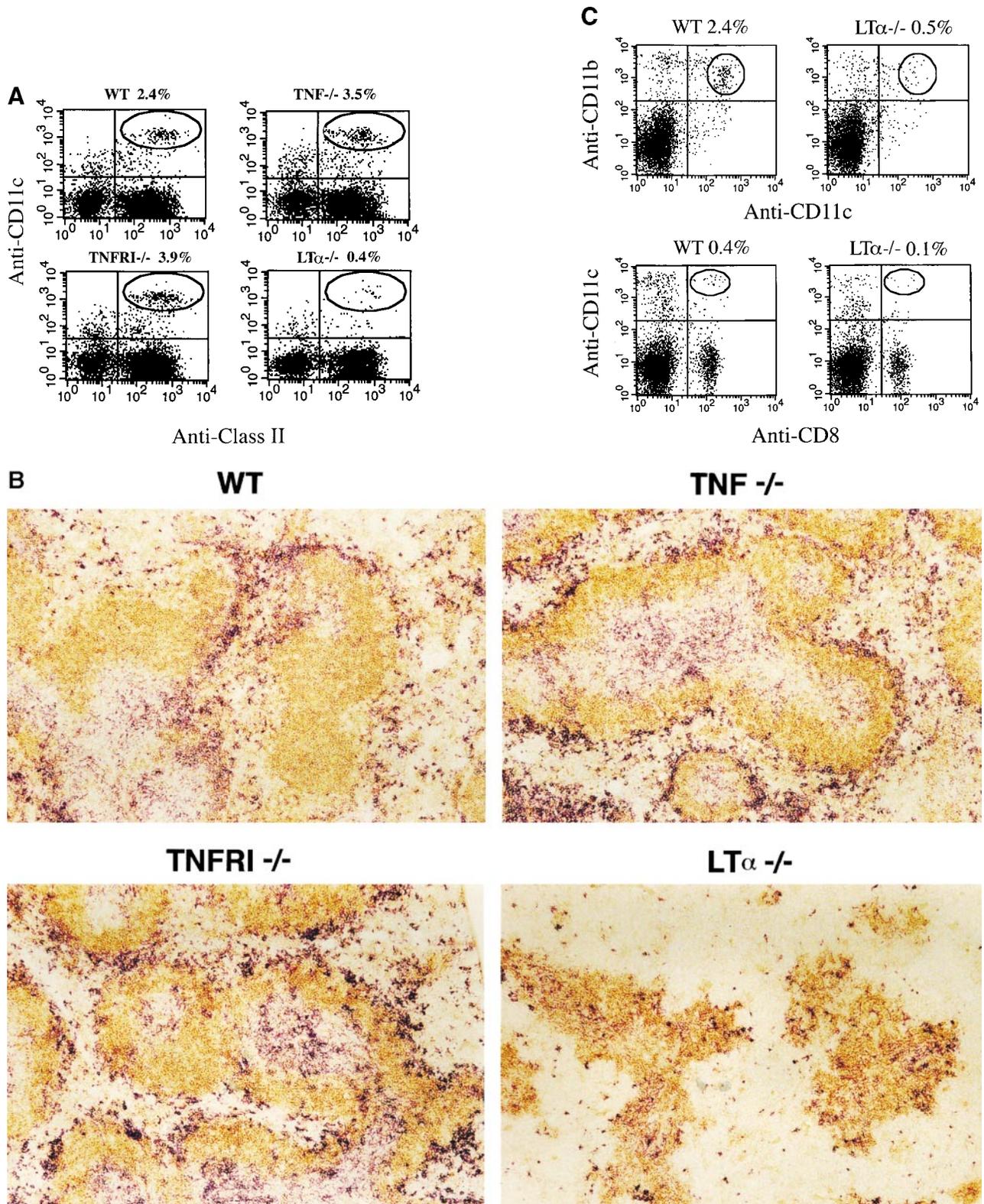
Figure 1. Determination of DC number in mice deficient in LTα, TNF, or TNFR. Spleens from 6–10-wk-old wt (WT), TNF$^{-/-}$, TNFR$^{-/-}$, and LTα$^{-/-}$ mice were collected. (A) Splenocytes were stained for the DC marker (CD11c) and class II marker (I-A^b) as indicated. (B) The frozen spleen sections were stained for anti-CD11c antibody for DC (red) and anti-B220 antibody for B cells (brown). Data from one of five representative experiments is shown. (C) Both lymphoid and myeloid DC subsets are reduced in LTα$^{-/-}$ mice. The splenocytes were stained for DC subsets with CD11b/CD11c and CD11c/CD8α as indicated.

Table I. *Regulation of Splenic DCs by Membrane LT but Not by Soluble LTα₃ or TNF-α₃*

Mice	Treatment*	Percentage of[‡] DCs	T cells	Presence of FDCs	T/B zones
wt	—	2.4 ± 0.4	32 ± 6	+	+
LTα$^{-/-}$	—	0.6 ± 0.2[§]	34 ± 3	−	−
TNFR-I$^{-/-}$	—	3.7 ± 0.4	27 ± 3	−	+
TNFα/$^{-/-}$	—	2.9 ± 0.5	32 ± 4	−	+
wt	LTβR–Ig	0.9 ± 0.2[§]	32 ± 8	−	+
wt	control Ig	2.6 ± 0.8	31 ± 9	+	+
RAG-1$^{-/-}$	LTβR–Ig	3.7 ± 0.2[§]	NA	−	−
RAG-1$^{-/-}$	control Ig	11 ± 0.2	NA	−	−

*A single dose (50–100 μg) was administered intraperitoneally, and the spleens from four to six mice were collected 10–15 d later for FACS staining. Total number of splenocytes is comparable in wt and LTα$^{-/-}$ mice (6).

[‡]Numbers represent cells with the indicated phenotypes expressed as mean percentage ± SD.

[§]Statistically significant differences ($P < 0.01$) between wt and LTα$^{-/-}$ mice or between the LTβR–Ig-treated group and the control Ig–treated group.

NA, not applicable due to the lack of T and B cells.

velopment of DCs in vivo, splenocytes from TNF$^{-/-}$ and wt mice were stained for CD11c and MHC class II (I-A^b), and the number of DCs in the preparation was determined by flow cytometry. The total number of DCs in both types of mice was similar, suggesting that TNF is not essential for the development of DCs (Fig. 1 A and Table I). Interestingly, the number of DCs in LTα$^{-/-}$ mice was greatly reduced, especially for the CD11c^{high}class II^{high} subset (Fig. 1 A and Table I), suggesting a role for LTα in DC development. Soluble LTα and TNF-α are structurally related homotrimers (LTα₃ and TNF-α₃) that exhibit similar biological activities by binding to the defined TNFRs (1), so TNFR$^{-/-}$ mice were used to determine the role of TNFR in DC development (Fig. 1 and Table I). However, the normal number of DCs in the spleens of TNFR$^{-/-}$ mice suggests that signaling via TNFR by either LTα₃ or TNF-α₃ is not essential for the presence of DCs in the spleen.

CD11c$^+$ DC subsets preferentially migrate to distinct areas in the spleen (18, 19): myeloid DCs (CD8α$^-$/CD11b$^+$) are mainly located in the marginal zones (MZs) of white pulp, whereas lymphoid DCs (CD8α$^+$/CD11b$^-$) are preferentially located in the T cell zones of white pulp. To study whether LT or TNF preferentially regulates a subset of DCs, the distribution of DCs and B cells in the spleens of TNF$^{-/-}$ mice and LTα$^{-/-}$ mice was visualized histologically (Fig. 1 B). Clusters of splenic DCs were readily observed in the T cell zone and MZ of wt and TNF$^{-/-}$ mice; however, only a few dispersed DCs were randomly present in the spleens of LTα$^{-/-}$ mice. The distribution

pattern and number of DCs visualized in situ closely correlated to that measured by flow cytometry, which showed that both myeloid and lymphoid DCs were proportionally reduced in LTα$^{-/-}$ mice (Fig. 1 C). Considering that myeloid and lymphoid DCs may be distinct populations of DC subsets (18, 19), it is interesting to notice that the presence of both subsets was regulated by LT.

Signaling via LTβR by Membrane LTα₁β₂ Is Required for the Presence of DCs. LTα$^{-/-}$ mice lack both soluble LTα₃ and membrane-associated LTα₁β₂, which bind to separate receptors, TNFR and LTβR, respectively (1, 2). As the number of DCs in TNFR-I$^{-/-}$ mice was similar to that in wt mice (Fig. 1 A and Table I), it was possible that membrane LTα₁β₂, instead of soluble LTα₃, was required for the presence of DCs in the spleen. To test this hypothesis, LTβR–Ig was used to block membrane LT activity in wt adult mice, which resulted in the absence of FDCs in 1 wk. Interestingly, the number of DCs but not lymphocytes in the spleens was markedly reduced 10 d after the administration of a single dose of LTβR–Ig (Fig. 2 and Table I). Moreover, the distribution pattern of the remaining DCs in the spleen was similar to that in LTα$^{-/-}$ mice.

Expression of LT has been detected primarily in activated T, B, and NK cells (1, 2). However, the percentage of DCs in the spleen of TCR$^{-/-}$BCR$^{-/-}$ CD3ε-transgenic mice or RAG-1$^{-/-}$ mice is not obviously reduced (data not shown). In fact, the percentage of DCs in the splenocytes of RAG1$^{-/-}$ mice is three- to fourfold higher than that of wt mice (Fig. 2, A and B). This suggests that the development of DCs could be independent of LT expression on T and B cells. To rule out whether the DC development observed in RAG-1$^{-/-}$ mice might be occurring via an LT-independent pathway, RAG-1$^{-/-}$ mice were treated with LTβR–Ig for 10 d (Fig. 2 and Table I). A significant reduction of splenic DCs (60–90% reduction) was readily detected, demonstrating that LT-expressing cells other than T and B cells control the migration of DCs (Table I). Although NK cells in RAG1$^{-/-}$ mice were plausible candidates for regulating DC migration in an LT-dependent pathway, RAG-1$^{-/-}$ mice depleted of NK cells (with 300 μg of PK136, an anti-NK1.1 antibody) did not exhibit reduced numbers of splenic DCs. Consistent with this data, no reduction of DCs was detected in CD3ε-transgenic mice lacking both NK and T cells. It is likely that cells other than T, B, and NK cells also express low levels of LT, regulating the migration of DCs.

Murine LTβR–Ig may block ligands other than membrane LT. It has been shown that human LTβR–Ig can also bind to human LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes), a recently identified membrane-associated TNF family member (26). The biological consequence of this binding is unclear. To exclude the potential effect of LIGHT, an anti–murine LTβ mAb, which specifically binds to the LTβ chain but not LIGHT, was administered to wt mice. Such treatment also resulted in a reduced number of DCs and their subsets similar to the ef-
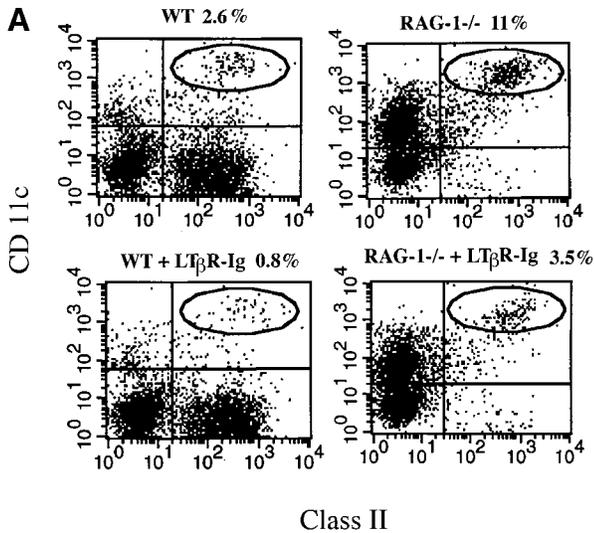
Figure 2. Reduced numbers of DCs in the mice treated with LTβR–Ig. Wild-type (WT) mice (left) and RAG-1$^{-/-}$ mice (right) were treated with 50 μg human LFA-3–Ig (top) or LTβR–Ig (bottom). The spleens were collected 10 d after treatment. (A) The number of splenic DCs determined by flow cytometry analysis. (B) Distribution of DC clusters determined by immunohistology. The frozen spleen sections from these mice were stained for anti-CD11c antibody (red) and anti-B220 antibody (brown). Data from one of six experiments is presented.
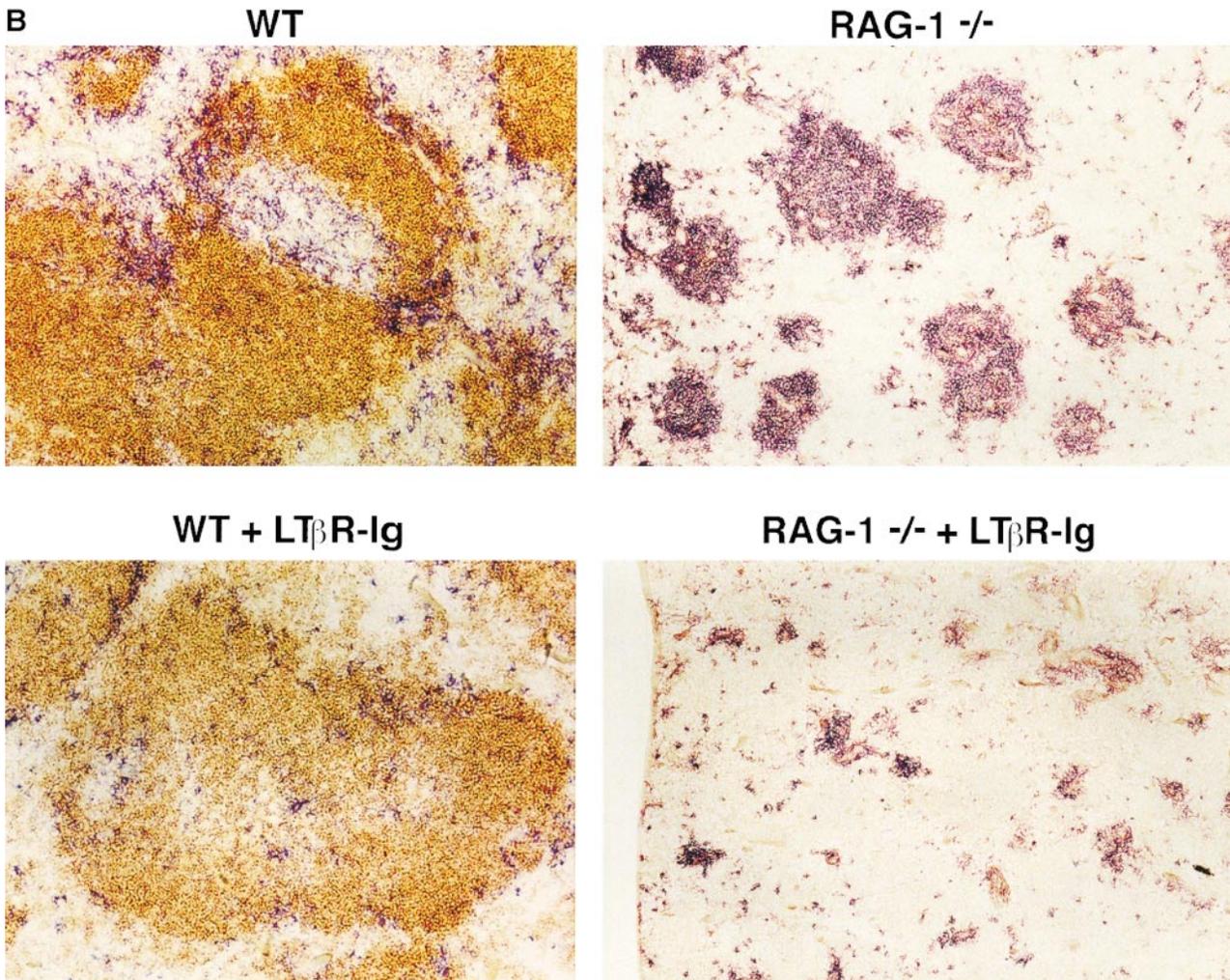
fect of LTβR–Ig (Fig. 3 A). Our data clearly indicate that LTα$_1$β$_2$ is the ligand required for the presence of DCs in the spleen. As ligands from the TNF family can bind to more than one receptor, the number of splenic DCs in LTβR$^{-/-}$ mice was determined to directly address whether signaling via LTβR is required for the presence of DCs in lymphoid tissue. The number of DCs in these mice was also lower than in wt mice (Fig. 3 B). Thus, the data strongly suggest that signaling via LTβR by membrane LT is essential for the presence of DCs in the spleen.
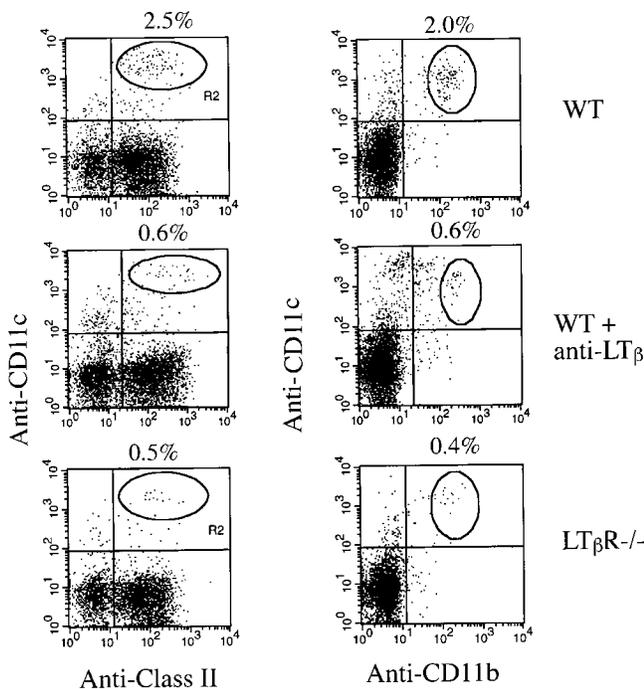
Figure 3. Signaling via LTβR by LTα$_1$β$_2$ is required for the presence of DCs in the spleen. Splenocytes from mice treated with anti-LTβ antibody or LTβR$^{-/-}$ mice were enzymatically treated and stained for the DC marker (CD11c) and class II marker (I-A^b) (left panels) or CD11c and CD11b (right panels).

Ineffective Migration of wt DCs into Spleens of LTα$^{-/-}$ Mice. Fewer DCs in the lymphoid tissues of mice lacking LT may be related to a reduction of DC progenitors in BM, impaired migration, or an accelerated removal of these cells. To test whether there was a deficiency in DC progenitors or the growth of DCs in LTα$^{-/-}$ mice, BM cells from either wt or LTα$^{-/-}$ mice were cultured by standard protocol using different doses of GM-CSF and IL-4 (25). The number of DC colonies and total number of DCs was comparable between wt and LTα$^{-/-}$ mice. In addition, the number of DC colonies from wt mice was not altered by coculture with LTβR–Ig (data not shown). Together, the data suggest that LT is not an essential survival factor or growth factor for DCs or their progenitors.

It has recently been shown that LT and, to lesser degree, TNF stimulates stromal cells to release chemokines, which may determine the migration or segregation of T and B cells in the spleen (16). It is possible that the migration of DCs into lymphoid tissues of LTα$^{-/-}$ mice is impaired due to the lack of LT-mediated chemokines for DCs. If the migration of DCs into lymphoid tissues is impaired in LTα$^{-/-}$ mice, the question would be where DCs accumulate in the absence of LT. If the BMDC development remains functional in the absence of LT, we would expect that the reduced number of DCs in lymphoid tissues in the absence of LT might be associated with an increased number of DCs in nonlymphoid tissues. Interestingly, there is an accumulation of lymphocytes around perivascular areas in lungs, liver, pancreas, submandibular glands, kidneys, and other tissues in LTα$^{-/-}$, LTβ$^{-/-}$, and LTβR$^{-/-}$ mice (7–9). To test whether the number of DCs was also increased in nonlymphoid tissues, DCs in lungs were quantitated in wt and LTα$^{-/-}$ mice. In contrast to the reduced number of DCs in lymphoid tissues, the number of DCs in lungs of LTα$^{-/-}$ mice was much higher than in wt mice (10.5 ± 1.8 × 10^5 vs. 2.9 ± 1.3 × 10^5). This suggests that LT is required for the proper distribution of DCs.
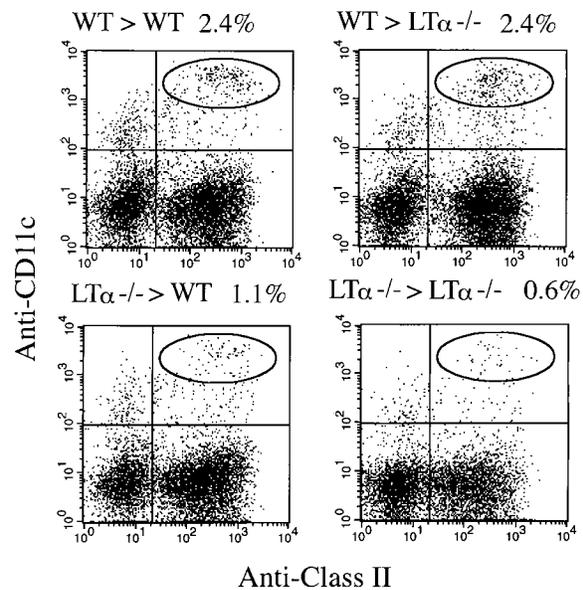
To directly study whether the migration of DCs into the spleen was impaired in LTα$^{-/-}$ mice, DCs expanded from the BM of Ly5.1 wt mice were transferred into LTα$^{-/-}$ and C57BL/6 mice (Ly5.2), respectively. The number of Ly5.1 DCs recovered from the spleens of wt mice was two- to fourfold higher than that from LTα$^{-/-}$ mice, although both groups received similar numbers of DCs from the same source (Fig. 4 A). Ly5.1$^+$CD11c$^-$ donor cells, mainly macrophages, in both groups were roughly the same (Fig. 4 A). As the number of splenic DCs in wt mice was not reduced within the first week after administration of a high dose of LTβR–Ig, it is unlikely that transfer of Ly5.1 DCs into LTα$^{-/-}$ mice leads to the premature death (<24 h) of these DCs.

It is possible that the splenic environment in LTα$^{-/-}$ mice did not allow the efficient sequestration or migration of DCs. The splenic environment essential for the localization of DCs may include its architecture, the size and shape of white pulps, and cytokines, such as chemokines, produced from the spleen. Altered splenic architecture and smaller white pulp in LTα$^{-/-}$ mice are readily visualized defects that may structurally impair the migration of splenic DCs into the proper area. However, short-term blockage of membrane LT by LTβR–Ig in wt mice had no detectable impact on the architecture or size of white pulps, yet this treatment still prevented the effective migration of DCs into the T cell zone and B cell follicles (Fig. 2 and Table I). This suggests that altered architecture itself is not the primary cause of reduced migration of DCs into the spleens of LTα$^{-/-}$ mice. Interestingly, the altered T/B cell segregation correlated with the altered localization of DCs (Fig. 2) and with altered chemokine production in the absence of LT (16).

To study whether additional membrane LT can restore the localization of DCs in the spleens of LTα$^{-/-}$ mice, we transferred LT-expressing lymphocytes and DCs from wt mice into LTα$^{-/-}$ mice. The altered splenic architecture remained, but the number of CD11c$^+$ cells in LTα$^{-/-}$ recipients was comparable to that in wt recipients 10 d after transfer (Fig. 4 B), again suggesting that the overall architectural defect in LTα$^{-/-}$ mice may not be the primary cause of reduced number DCs in the spleen. It appears that the microenvironment in the spleen required for the presence of DCs is rather flexible and can be altered in 1–2 wk. Interestingly, the timing of the reduction of DCs is also consistent with the maximum reduction of various chemokines in the spleen 1–2 wk after administration of LTβR–Ig (16). Thus, the data suggest that the reduced number of DCs in LTα$^{-/-}$ mice may be due, at least in

A

0.32%   0.14%

Anti-CD11c

Anti-Ly5.1

B

WT > WT

WT > LTα-/-

LTα-/- > LTα-/-
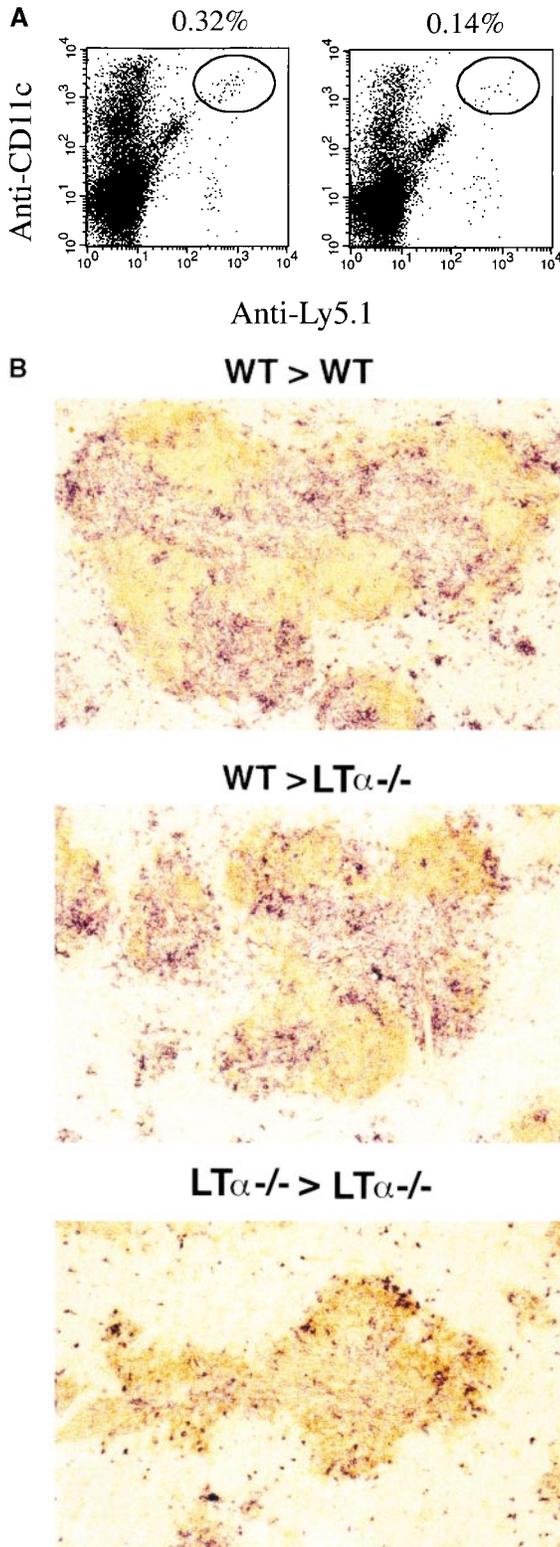
Figure 4. Migration of wt DCs into the spleens of LTα$^{-/-}$ mice in the absence or presence of LT-expressing cells. (A) Impaired migration of DCs in LTα$^{-/-}$ mice. BMDCs (5×10^6) from Ly5.1 mice expanded in vitro in the presence of GM-CSF and IL-4 were transferred to sublethally irradiated LTα$^{-/-}$ mice (right) and C57BL/6 mice (left). The spleens were collected 24 h later and stained for Ly5.1 and CD11c. One of four experiments is represented. CD11c$^-$ cells from donor origin are comparable in both groups (∼0.25%). (B) Restoration of the splenic DCs in

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WT > WT 2.4%   WT > LTα-/- 2.4%

LTα-/-> WT 1.1%   LTα-/- > LTα-/- 0.6%

Anti-CD11c

Anti-Class II

Figure 5. Determination of the distribution of splenic DCs by BM-derived cells in an LT-dependent fashion. BM reconstitution was performed according to previously described methods (12). 6 wk after BM reconstitution, spleens were collected and treated by using collagenase (24). The cells were stained for anti-CD11c and anti–class II antibody. Data from one of three experiments is presented.

part, to the impaired migration of DCs that may be mediated through altered chemokine production. The nature of the LT-responsive stromal cells and the exact type of chemokines remains to be determined.

LTα-mediated Microenvironment that Permits the Migration of DCs Is Determined by BM-derived Cells. BM transfer in long-term reconstitution provides a model to evaluate the role of LTα in determination of the splenic microenvironment that permits the migration of DCs. 6 wk after lethally irradiated LTα$^{-/-}$ mice were reconstituted with wt BM, DCs were restored to a level similar to that seen in irradiated wt mice reconstituted with wt BM (Fig. 5). This suggests that the altered microenvironment that impairs the migration of DCs is not developmentally fixed and that LT-expressing BM cells could restore the migration of DCs. In contrast, when lethally irradiated wt mice were reconstituted with LTα$^{-/-}$ BM, the number of DCs in the spleen was reduced, as is seen in LTα$^{-/-}$ mice or LTβR–Ig-treated mice (Fig. 5). Therefore, the LTα-mediated microenvironment that permits the migration of DCs is primarily determined and maintained by LT-expressing BM-derived cells.

Impaired Mixed Leukocyte Reaction in LTα$^{-/-}$ Mice. To examine whether reduced numbers of DCs in lymphoid tissues of LTα$^{-/-}$ mice could impair the overall function

LTα$^{-/-}$ mice 10 d after cotransfer of LT-expressing splenocytes. 5×10^7 splenocytes from wt mice prepared by gentle pressure through a cell strainer were transferred (>) into sublethally irradiated wt (WT) or LTα$^{-/-}$ mice as previously reported (12). The spleens were harvested 10 d after transfer, and frozen sections were stained with anti-B220 (brown) and anti-CD11c (red) to visualize the number and distribution of DCs.
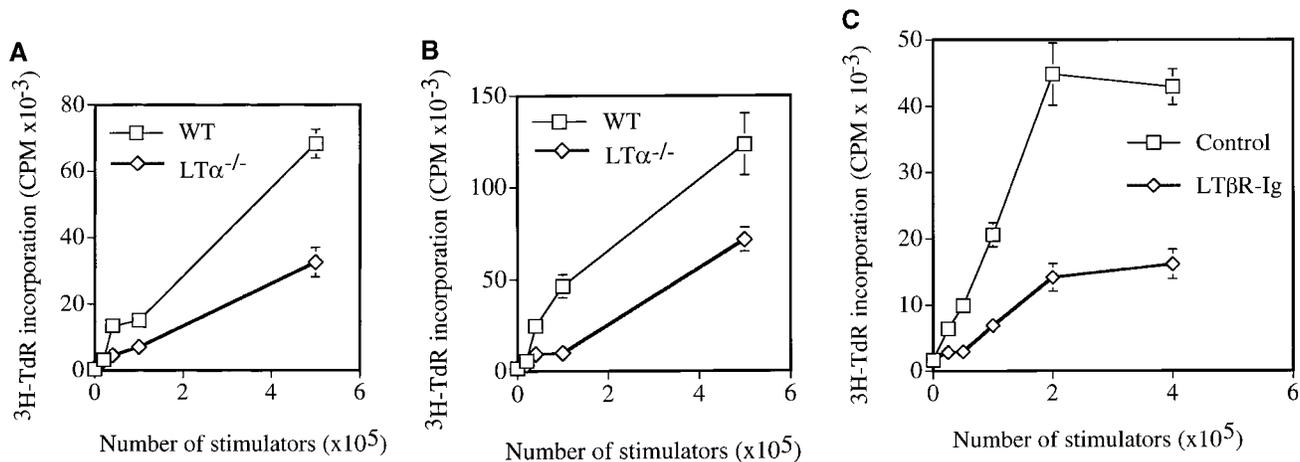
Figure 6. Impaired ability to stimulate allogenic T cells by splenocytes from LTα$^{-/-}$ mice or wt mice treated with LTβR–Ig in mixed lymphocyte reaction assay. Responder LN cells from BALB/c mice were cultured with a titration of irradiated splenocytes from C57BL/6 or LTα$^{-/-}$ mice prepared by mechanic pressure (A), collagenase digestion (B), or from mice pretreated with LTβR–Ig for 10 d (C). Data from one of four experiments is presented.

of DCs, the ability of DCs in LTα$^{-/-}$ mice to stimulate allogenic T cells was evaluated by mixed leukocyte reaction (MLR). Mechanically separated splenocytes from LTα$^{-/-}$ mice showed a decreased ability to stimulate allogenic T cells in a dose-dependent manner (Fig. 6 A). To rule out the possibility that reduced antigen-presenting activity in the splenocytes of LTα$^{-/-}$ mice is associated with the failure to release DCs from altered architecture of the spleen using physical separation, spleen fragments from both LTα$^{-/-}$ mice and wt mice were subjected to collagenase digestion to release DCs. The collagenase-treated splenocytes from LTα$^{-/-}$ mice showed profound defects (four- to eightfold lower) in antigen-presenting activity compared with those from wt mice, especially when the total splenocytes was in the range of 0.2–1 × 10^5 cells (Fig. 6 B). To exclude the impact from either of the developmental defects in LTα$^{-/-}$ mice, the splenocytes from LTβR–Ig-treated C57BL/6 mice were collected by mechanical pressure and used as stimulators. Severalfold reductions of radiation count were readily detected in the LTβR–Ig-pretreated group, as in the case of LTα$^{-/-}$ mice (Fig. 6 C). In general, the lower MLR closely correlated with the lower number of DCs (Figs. 1, 2, and 6). The number of other potential APCs, such as B cells, in the spleens of LTα$^{-/-}$ mice or mice treated with LTβR–Ig appears to be comparable to that in wt mice. It was proposed a decade ago that DCs are the principal stimulators of MLR in the spleen (27, 28); our results further support the proposal, as reduced numbers of DCs in LTα$^{-/-}$ mice could account for the impaired MLR.

Our results have revealed that membrane LT and LTβR are the natural ligand–receptor pair essential for the presence of splenic DCs in vivo. LTα$^{-/-}$ mice exhibit reduced numbers of DCs in the spleen, whereas both TNF$^{-/-}$ and TNFR$^{-/-}$ mice show normal numbers of splenic DCs, suggesting that signaling via TNFR by either soluble LTα or TNF is not an essential pathway for the regulation of

DCs in the spleen. The notion that membrane LT is an essential ligand for the presence of DCs in the spleen is further supported by the reduced number of DCs in the wt spleen after the administration of either LTβR–Ig or anti-LTβ mAb. The results also suggest that signaling via LTβR by membrane LT is required for the presence of DCs, as LTβR is the only identified receptor for membrane LT. Finally, the lower number of splenic DCs in LTβR$^{-/-}$ mice confirms our hypothesis. In terms of the regulation of development or migration of DCs in the spleen, an essential role of either soluble LTα$_3$ or TNF-α$_3$ has not been demonstrated. However, TNF-α$_3$ or LTα$_3$ can coordinate membrane LTα$_1$β$_2$ in the development of lymphoid tissues (2, 10) and also may play a minor role in the migration of DCs in some situations. Interestingly, recent studies reported that high levels of soluble LTα$_3$ were able to induce chemokines and adhesion molecules in vitro (29). Ectopic expression of LTα$_3$ induces lymphocyte infiltration in non-lymphoid tissue, suggesting that the overexpression of LTα$_3$ may still play a role in the migration of some lymphoid cells (30–32). Ectopic LT in LTα$^{-/-}$ (RIPLT.LTα$^{-/-}$) mice also restored some LN, but a decreased number of interdigitating DCs was apparent in the LN (31). Therefore, proper expression of LT in the LN may also be required for the presence of DCs in the LN.

The ineffective migration of DCs may account for the reduced number of DCs in the spleens of mice lacking membrane LT or its receptor: (a) compared with wt recipients, fewer donor DCs were present in the spleens of LTα$^{-/-}$ recipients; (b) a reduced number of DCs is not developmentally fixed and can be repaired by LT-expressing cells; (c) the timing of altered numbers of DCs is consistent with the altered expression of chemokines in the spleen; (d) no significant impairment of DC growth or reduced DC progenitors can be detected; and finally, (e) DCs accumulate in nonlymphoid tissues in both LTα$^{-/-}$ and LTβR$^{-/-}$ mice, strongly supporting our notion that the

reduced number of DCs in the spleen is caused by impaired migration. Interestingly, fewer randomly distributed DCs in the spleens of LTBR–Ig-treated mice could still move to the T cell zone after intravenous injection of LPS, suggesting that fine positioning of DCs in the spleen could be regulated in an LT-independent fashion.

A number of chemokines are constitutively secreted in the lymphoid organs in an LT-dependent fashion (16). Altered distribution of T cells, B cells, and DCs in vivo may be regulated by some chemokines. Whether proper distribution of DCs and FDCs will facilitate T/B cell segregation remains to be determined. Although the expression of several chemokines has been found to be downregulated in the absence of LT, the exact chemokine that is essential for the migration of DCs has yet to be identified. Which chemokines are upregulated for directing DCs into nonlymphoid tissues in the absence of LT is completely unknown. Interestingly, the migration of most subsets of macrophages in the spleen is largely unchanged in the absence of LT (Fig. 4 A), suggesting that the chemokines that regulate the distribution of DCs may be distinct from those that regulate the distribution of macrophages. It will be important to determine whether the differences in the migration patterns of macrophages and DCs may account for differences in their biological activities. In addition to the action of LT on stromal cells, it is also possible that direct signaling via LTβR on DCs by membrane LT is required for the migration of DCs in the spleen.

Reduced numbers of DCs may account for reduced MLR, which is a DC-based T cell response. However, migration of DCs into lymphoid tissues for systemic immune responses may be more important for the generation of immune responses in vivo. In fact, after capturing antigens outside lymphoid tissues, DCs must migrate into lymphoid tissues to prime rare antigen-specific lymphocytes, which constantly recirculate through peripheral lymphoid tissues (18, 19). Regulation of the migration of DCs may provide an additional means to manipulate immune responses, T cell responses in particular. Consistent with that notion, we have found that inhibition of membrane LT has profound effects in several T cell–based disease models. For example, administration of LTβR–Ig reduced severity of colitis (17), collagen-induced arthritis, and experimental autoimmune encephalitis (J.L. Browning, unpublished observation). Clearly, the membrane LT/LTβR system provides an interesting model to further study DC biology and DC-mediated diseases.

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