Essential Role of Lymph Nodes in Contact Hypersensitivity Revealed in Lymphotoxin-α–deficient Mice

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Abstract

Lymph nodes (LNs) are important sentinal organs, populated by circulating lymphocytes and antigen-bearing cells exiting the tissue beds. Although cellular and humoral immune responses are induced in LNs by antigenic challenge, it is not known if LNs are essential for acquired immunity. We examined immune responses in mice that lack LNs due to genetic deletion of lymphotoxin ligands or in utero blockade of membrane lymphotoxin. We report that LNs are absolutely required for generating contact hypersensitivity, a T cell–dependent cellular immune response induced by epicutaneous hapten. We show that the homing of epidermal Langerhans cells in response to hapten application is specifically directed to LNs, providing a cellular basis for this unique LN function. In contrast, the spleen cannot mediate contact hypersensitivity because antigen-bearing epidermal Langerhans cells do not access splenic white pulp. Finally, we formally demonstrate that LNs provide a unique environment essential for generating this acquired immune response by reversing the LN defect in lymphotoxin-$\alpha^{-/-}$ mice, thereby restoring the capacity for contact hypersensitivity.

Key words: haptens • antigens • Langerhans cells • lymphoid tissue • spleen

Introduction

Secondary lymphoid organs are sites of immunological coordination, where diverse cell types, including T cells, B cells, APCs, and follicular dendritic cells (FDCs),[1] are brought together within a highly structured, organized environment. This organization has evolved to maximize antigen presentation to the rare T cells capable of specific TCR-mediated antigen recognition and clonal expansion, and is considered necessary for the efficient development of immune responses (1–3). Examples of secondary lymphoid organs include the spleen, LNs, Peyer's patches (PPs), mucosa-associated lymphoid tissues (MALTs), and lymphocyte aggregates such as the follicles and cryptopatches of the intestines and colon. These lymphoid compartments share striking similarities in cellular organization, such as discrete T and B cell areas, yet each also has unique, specialized portals for cell or antigen entry, e.g., the afferent lymphatics and high endothelial venules (HEVs) of LNs, the marginal zone (MZ) of the spleen, and the M cell–rich dome of PPs. LNs are found throughout the body, yet despite intensive investigation of the role of LNs in cellular and humoral immune responses, it has not been demonstrated whether these organs are absolutely required for any specific immune function.

In this study, we investigate whether LNs are required to generate a T cell–dependent cellular immune response, contact hypersensitivity (CH). It has been recognized for

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[1]*Abbreviations used in this paper:* BM, bone marrow; CH, contact hypersensitivity; DC, dendritic cell; DNFB, dinitrofluorobenzene; ELC, EBV-induced molecule 1 ligand chemokine; FDC, follicular dendritic cell; LC, Langerhans cell; LT, lymphotoxin; mLN, mesenteric LN; MZ, marginal zone; pLN, peripheral LN; *plt*, paucity of LN T cells; PP, Peyer's patch; SLC, secondary lymphoid tissue chemokine; TMA, phenyltrimethylammoaniline.

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many years that efficient epidermal immune responses depend on Langerhans cell (LC) uptake of antigen and traffic to LNs (4, 5). However, studies directly addressing the role of LNs in CH responses have used either physical disruption of LN lymphatic connections (6, 7), or agents capable of blocking LCs or T cell access to LNs (8, 9), thereby isolating antigen and responding cells. These studies showed that the CH response is not primed in situ, i.e., in the skin itself, and, along with the observation that LCs arrest in draining LNs (10), suggested that LNs are sufficient to mediate CH responses. Although these data demonstrate a correlation between LC traffic to LNs, intact lymphatic vasculature, and CH reactions, it remains unclear if LNs are the sole, and therefore essential, site where T cell priming to epidermal antigens occurs. Therefore, we have developed novel model systems to test whether LNs are absolutely required for CH responses.

Here we utilize lymphotoxin (LT)$\alpha^{-/-}$ and LT$\beta^{-/-}$ mice, which have developmental defects in LN genesis, to study whether LNs are essential to generate a CH response. Interaction of the membrane-bound, heteromeric ligand LT$\alpha\beta$ with its receptor (LTβ-R) is a critical signal for the genesis of LNs and PPs during embryonic development (3, 11, 12). The LT$\alpha^{-/-}$ mouse is essentially devoid of LNs and PPs (13, 14). The LT$\beta^{-/-}$ mouse has a similar phenotype; however, these mice can retain a subset of mucosa-associated LNs (15, 16). LT gene–deficient mice thus provide potentially useful models for investigating the role of LNs in various immune responses. However, the phenotypes of these knockout mice include additional alterations in the immune system (e.g., loss of the splenic MZ and of FDC networks in spleen and LNs [3, 17–21]) which lead to the disruption of splenic germinal center formation and Ab responses (3). Therefore, we have employed in utero manipulation to induce LN formation in LT$\alpha^{-/-}$ mice and to block LN formation in wild-type mice (12, 22, 23), to create model systems which allow us to determine if the absence of LNs, or the loss of LT ligand expression, impacts the CH response. Our data clearly demonstrate that epidermal CH responses are not generated in the absence of LNs, illustrating an essential role for LNs in this immune response. The specific trafficking of LCs to LNs is shown to provide a cellular basis for this unique LN function. These results have important implications for our understanding of LN function in local immunity and the trafficking of LCs to different lymphoid compartments.

Materials and Methods

CH and LC Trafficking Studies. All animal studies were performed in accordance with Institutional Animal Care and Use Committee standards. For LC trafficking studies, LT$\alpha^{-/-}$, LT$\beta^{-/-}$, LT$\alpha^{+/+}$, or wild-type mice were painted on the shaved skin of the upper back with 100 μl of 5 mg/ml FITC (Sigma-Aldrich) dissolved in 50% acetone/50% dibutylphthalate (Sigma-Aldrich). LNs were removed the next day for analysis of LC content by FACS® analysis and immunohistochemistry.

For CH experiments, dose response data were generated for each hapten using wild-type Balb/c and C57Bl/6 mice. The amount of hapten applied at both the sensitization phase and elicitation phase was titered to arrive at a dose that induced maximal response without excessive hapten. This was done to reduce the possibility that excess vehicle application could cause penetration beyond the epidermis, or induce other unanticipated responses, such as hapten tolerance (5, 24, 25). Mice were sensitized, rested for 7 d, then rechallenged on the ear. Caliper measurements of the ear were taken just before rechallenge and 24 h afterward, and the data calculated as the percent increase in thickness. For the hapten FITC, a dose of 100 μl of 5 mg/ml at sensitization and 5 μl of 5 mg/ml at elicitation gave a maximal response. Using the hapten 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazalone) dissolved in ethanol, a dose of 100 μl of 0.3% at sensitization and 10 μl 0.3% at elicitation was sufficient to elicit a maximal response. For the dinitrofluorobenzene (DNFB) studies, mice were painted on the shaved belly using 10 μl 1% solution in vehicle (4:1 acetone/olive oil) followed by 10 μl on the footpad 5 d later, a protocol similar to those successfully used in several recent studies (8, 9). Swelling of the footpad was measured after 24 h (challenged site − control site, in mm). Croton oil (ICN Biomedicals) was diluted to 0.8% in acetone. 10 μl was applied to each ear, and the swelling response was measured after 16 h. Phenyltrimethylammoaniline (TMA) was obtained from Bachem Inc. For experiments using TMA, mice were sensitized on the shaved abdomen either by subcutaneous injection or skin painting with 50 μl of a 10 mM solution in saline (subcutaneous) or DMSO (painting). Elicitation of the footpad was with 20 μl TMA presented either subcutaneously or by painting. Footpad swelling is measured as thickness at site of challenge − thickness of contralateral control.

Adoptive transfer experiments were performed essentially as described (9). In brief, Balb/c mice treated in utero with LTβ-R-Ig (see below) to ablate peripheral LN (pLN) and PP development were sensitized with oxazalone. 4 d later the mesenteric LN (mLN) and spleen were removed, and cells were isolated by mechanical disruption and red blood cell lysis. mLN cells isolated from untreated Balb/c mice were used as a negative control. 2.5×10^7 isolated cells were injected intravenously into normal, naive, syngeneic recipients, which were immediately challenged by ear painting with oxazalone. 24 h later the percent increase in ear swelling was measured. The results were compared with the CH response induced using the normal challenge/sensitization regimen.

In all experiments, mice were housed individually to prevent grooming of the sensitized area of the skin. All mice were coded and were measured in random order. Statistical significance of the change in ear thickness was determined using tests of the equivalence of the means.

Irradiation and Bone Marrow Transfer. LT$\alpha^{+/+}$ and LT$\alpha^{-/-}$ mice were lethally irradiated using 1,100 rad (Gamma cell; Nordion International Inc.) and reconstituted within 4 h with 2×10^6 bone marrow (BM) cells derived from the donor mice. The BM cell suspension was subjected to depletion using anti-Thy1.2 and rabbit complement (Accurate Chemical & Scientific Corp.) before use to remove mature T cells. After reconstitution, <5% of hemopoietic cells were recipient derived.

LN Induction and Ablation Protocols. To generate peripheral and mucosal LNs in the progeny, pregnant female LT$\alpha^{-/-}$ mice were injected with 250 μg of agonist anti–LTβ-R mAb AF.H6 via the tail vein beginning on gestation day 11 or 12, followed by additional injections intravenously every 48 h, as described (12). Other pregnant LT$\alpha^{-/-}$ mice were treated with control hamster mAb Ha4/8 (anti-KLH). To ablate LN development, pregnant

Table I. *Titration of the Hapten Doses Used in CH Experiments*

Hapten	Sensitization dose	Elicitation dose	CH response*
FITC-isothiocyanate	Fixed concentration‡ (μl)		
	200	10	65 ± 17
	100	10	100 ± 26
	50	10	39 ± 21
	25	10	20 ± 15
	0	10	0 ± 6
Oxazalone	Fixed volume§ (%)		
	1.0	0.3	109 ± 7
	0.3	0.3	107 ± 31
	0.3	0.1	93 ± 27
	0.1	0.1	79 ± 23
	0	0.3	12 ± 12

Mice were shaved and painted on the upper trunk with the indicated doses of hapten.
*5 (oxazalone) or 10 (FITC) d later mice were rechallenged on the ears, and the percent increase in the thickness of the ears was measured after 24 h.
‡For FITC experiments, a concentration of 5 mg/ml was used, and the volume was varied.
§For oxazalone experiments, fixed volumes of 150 μl at sensitization and 10 μl at elicitation were used, and the concentration of hapten was varied.

female Balb/c or C57Bl/6 mice were injected intravenously multiple times with 100 μg of purified murine LTβ-R-Ig, 100 μg TNF-R55-Ig, or both together, as described (12). The presence or absence of LNs was carefully determined by dissection of representative progeny of each litter, and confirmed in each individual animal at necropsy.

Immunohistochemistry and FACS® Analysis. Mouse spleen and LNs were harvested, frozen, sectioned, and stained for B220 and CD11c as described previously (22, 23). Trafficking of LCs to LNs was analyzed by UV induced fluorescence of the captured FITC, in combination with PE-coupled anti-B220 mAb (BD PharMingen). All sections were viewed under 50× or 100× optics and captured as digitized image files using either single or dual filters. The images shown are representative of those obtained from a minimum of four animals per treatment group from a minimum of four independent experiments. The identity of FITC-laden LCs in LNs was confirmed using FACS® analysis and CELLQuest™ software (Becton Dickinson).

Results

LTα$^{-/-}$ Mice Cannot Mount a CH Response to Haptens. Immunohistochemical staining revealed that the distribution of LCs in the skin of LTα$^{-/-}$ mice was essentially indistinguishable from wild-type mice (unpublished data). Therefore, we tested if these LCs were capable of supporting CH responses in the absence of LNs. We first established dose response curves for the response of wild-type mice to the haptens FITC and oxazalone (Table I) and confirmed that our established doses were within the linear portion of the

Table II. *LTα$^{-/-}$ Mice Cannot Mount a CH Response to Secondary Hapten Challenge but Retain a Sensitivity Response to Hapten Presented Subcutaneously*

CH responses				Subcutaneous and CH responses to TMA			
Strain	Sensitization	Elicitation	Swelling (%)	Strain	*n*	Priming dose and route	Swelling (mm)
LT$^{+/+}$	FITC	Vehicle	9 ± 3*	LTα$^{+/-}$	5	10 mm, 50 μl s.c.	0.90 ± 0.23
LT$^{+/+}$	FITC	FITC	61 ± 8	LTα$^{+/-}$	6	10 mm, 50 μl s.c.	0.70 ± 0.12
LTα$^{-/-}$	FITC	FITC	16 ± 8*	Control	3	0	0.18 ± 0.02
LT$^{+/+}$	Vehicle	0.3% oxazalone	5 ± 8.5*	LTα$^{-/-}$	5	10 mm, 50 μl e.c.	0.68 ± 0.16
LT$^{+/+}$	0.3% oxazalone	0.3% oxazalone	31.6 ± 13.6	LTα$^{-/-}$	6	10 mm, 50 μl e.c.	0.28 ± 0.11‡
LTα$^{-/-}$	0.3% oxazalone	0.3% oxazalone	5.1 ± 4.3*	Control	3	0	0.10 ± 0.03
LT$^{+/+}$	2% oxazalone	0.3% oxazalone	40 ± 22				
LTα$^{-/-}$	2% oxazalone	0.3% oxazalone	4.7 ± 6.1*				
LT$^{+/+}$	None	0.8% croton oil	23.5 ± 2.8				
LTα$^{-/-}$	None	0.8% croton oil	31.5 ± 9.7				

For skin sensitization, mice were painted with 100 μl hapten. 10 d later, CH was elicited by painting the ears with 10 μl hapten. Ear thickness was measured and the average percent increase in thickness ($n = 6$, FITC; $n = 8$, oxazalone; $n = 4$, croton oil) was determined for each set of mice. FITC and oxazalone data are representative of four independent experiments. Croton oil responses were not statistically different from each other. For experiments using TMA, mice were sensitized on the shaved abdomen with 50 μl either by subcutaneous (s.c.) injection or skin painting (e.c.). Elicitation of the footpad was with 20 μl TMA presented either subcutaneously or by painting. Footpad swelling is thickness at site of challenge − thickness of contralateral control. The TMA experiments were performed three times with similar results.
*$P < 0.005$ for values significantly less than LT$^{+/+}$/hapten positive control.
‡$P < 0.01$ for values significantly less than LT$^{+/+}$/hapten positive control.

Table III. *BM-derived Cells Are Not Responsible for the Defect in CH Response in LTα$^{-/-}$ Mice*

Experiment	Donor	Recipient	Priming dose	Footpad swelling
			%	*mm*
1	+/+	+/+	0.5	0.97 ± 0.09
	+/+	LTα$^{-/-}$	0.5	0.13 ± 0.06*
	LTα$^{-/-}$	+/+	0.5	0.75 ± 0.07
	LTα$^{-/-}$	LTα$^{-/-}$	0.5	0.08 ± 0.02*
Nonirradiated control		+/+	0.5	1.14 ± 0.17
		LTα$^{-/-}$	0.5	0.18 ± 0.06*
		+/+	0	0.17 ± 0.05*
2	+/+	+/+	1.0	0.92 ± 0.27
	+/+	LTα$^{-/-}$	1.0	0.07 ± 0.02*
	LTα$^{-/-}$	+/+	1.0	0.52 ± 0.12
	LTα$^{-/-}$	LTα$^{-/-}$	1.0	0.09 ± 0.04*
Nonirradiated control		+/+	0	0.06 ± 0.01*
Nonirradiated control		LTα$^{-/-}$	0	0.09 ± 0.02*

Mice were lethally irradiated (1,100 rad) and reconstituted within 4 h with 2×10^6 BM cells from the indicated mice. Mature T cells in the BM cell suspension were depleted using anti-Thy1.2 mAb plus complement. The CH experiments were performed 6 wk later, using DNFB as the hapten, and three mice per group. Footpad swelling is thickness at site of challenge − thickness of contralateral control.
*$P < 0.0005$ for values less than the donor/recipient positive control.

response. Then, we initiated our studies of CH responses by comparing the ability of LTα$^{-/-}$ mice and LT$^{+/+}$ mice to respond to hapten challenge. In LTα$^{-/-}$ mice, the CH response to FITC was greatly reduced, approaching background levels (Table II). This result was independent of the hapten used, as LTα$^{-/-}$ mice were also unable to respond to oxazalone (Table II) and DNFB (Table III).

In experiments using another hapten (TMA), we observed that the delayed type hypersensitivity (DTH) response of LTα$^{+/+}$ and LTα$^{-/-}$ mice to subcutaneous injections were indistinguishable (Table II). This suggested that dermal APCs were triggering T cell activation despite the lack of LNs or of LT ligands. We tested if excessive hapten doses applied to the epidermis would breach epidermal integrity and lead to a mixed epidermal/dermal CH/DTH response. LTα$^{-/-}$ mice sensitized with as much as 150 μl 2% oxazalone failed to respond to subsequent challenge (Table II). Similarly, LTα$^{-/-}$ mice sensitized with as much as 20 μl of 1% DNFB did not respond to subsequent challenge (data not shown). Thus, LTα$^{-/-}$ mice did not mount a CH response even when excessive doses of hapten were used for sensitization. Finally, we examined the ability of LTα$^{-/-}$ mice to respond to chemical irritation using a croton oil assay. The response to irritant in this model is T cell independent (24). In contrast to the defect seen in the CH models, the irritant response of LTα$^{-/-}$ mice was similar to wild type (Table II). Therefore, LTα$^{-/-}$ were capable of an epidermal inflammatory response which does not require T cell activation.

BM-derived Cells from LTα$^{-/-}$ Mice Are Capable of Mediating CH Responses. The initial experiments in LTα$^{-/-}$ mice suggested that the CH response to epidermal antigen was defective despite normal T cell priming and immune response to antigens presented within the dermis. To assess whether the inability of lymphocytes to make surface LTαβ or secrete LTα was responsible for the defective CH response in LTα$^{-/-}$ mice, BM cells from LTα$^{-/-}$ mice were transferred into irradiated wild-type mice. Mice were used in CH experiments 8–12 wk after BM transfer. Such chimeric mice, which retain a full complement of LNs, had a slightly attenuated response to the hapten DNFB (Table III), indicating that LT expression by lymphocytes does not

Table IV. *LTα$^{-/-}$ Mice Treated In Utero with Anti–LTβ-R to Induce LN Genesis Have a Normal CH Response to Hapten*

Strain	Treatment in utero	LN formed	Sensitization	Elicitation	Percent swelling		
					Expt. 1	Expt. 2	Expt. 3
LTα$^{-/-}$	None or Ha4/8	None	FITC	FITC	14 ± 9*	nd	9 ± 7*
LTα$^{-/-}$	AF.H6	Brachial, axillary, mandibular, iliac, mesenteric, cervical, sacral	FITC	FITC	40 ± 19	37 ± 7	23 ± 7
LTα$^{+/+}$	None	All	FITC	FITC	40 ± 13	34 ± 10	25 ± 11
LTα$^{+/+}$	None	All	FITC	Vehicle	5 ± 1*	1 ± 4*	1 ± .4*

For sensitization, mice were painted with 100 μl FITC. 10 d later, CH was elicited by painting the ears with10 μl FITC. Ear thickness was measured and the average percent increase in thickness ($n = 6$) was determined for each set of mice. Expt., experiment; nd, not done.
*$P < 0.005$ for values significantly less than LT$^{+/+}$/FITC positive control.

Table V. *The Loss of LNs from LTβ$^{-/-}$ Mice Results in the Loss of the CH Response to Hapten*

Strain	LN found	Sensitization	Elicitization	Percent swelling		
				Expt. 1	Expt. 2	Expt. 3
LT$^{+/+}$	All	Vehicle	FITC	nd	3.1 ± 3.2*	2.5 ± 2.9*
LT$^{+/+}$	All	FITC	Vehicle	3.2 ± 3.8*	nd	2.3 ± 2.7*
LT$^{+/+}$	All	FITC	FITC	52.2 ± 20	24 ± 9.5	23 ± 11
LTβ$^{-/-}$	Mucosal	FITC	FITC	62.2 ± 3.3	29 ± 8.4	43 ± 18
LTβ$^{-/-}$	None	FITC	FITC	8.8 ± 2.7*	8.5 ± 4.4* (n = 2)	9.3 ± 6.8*

Mice were painted on the upper trunk with 100 μl FITC then rechallenged 10 d later on the ears with 10 μl FITC.
Ear thickness was determined and values shown are percent increase. "Mucosal" refers to the presence of one or more mesenteric, cervical, or sacral LNs. n = 6 in each experiment shown, except LTβ$^{-/-}$ mice, no LNs, in experiment 2 (n = 2 as noted). Expt., experiment; nd, not done.
*$P < 0.005$ for values significantly less than LT$^{+/+}$/FITC positive control.

play a dominant role in the CH response. Immunohistochemical analyses showed that no FDCs, germinal centers, or MZ markers could be detected in the spleen of these chimeric mice, indicating that LTα expressing cells were absent as expected (3).

When wild-type BM was transferred into irradiated LTα$^{-/-}$ mice and tested in the DNFB CH model, the chimeric mice were unable to respond to DNFB challenge (Table III). The wild type into LTα$^{-/-}$ BM transfer does not restore LN development, as LN organogenesis is developmentally regulated (12, 22); however, splenic function is restored (3). These results show that expression of LTα by circulating cells is not essential for CH, but rather suggests a critical role for LNs in the generation of the CH response.

FITC-mediated CH Is Restored in LTα$^{-/-}$ Mice that Have LNs. The BM transfer experiments suggested that LNs were required for CH responses. To formally address this hypothesis, we induced LN genesis in LTα$^{-/-}$ mice by using the agonist anti–LTβ-R mAb AF.H6 to signal LTβ-R during development (12). Progeny of litters which received this maternal transfer treatment were examined for the presence of LNs, and LTα$^{-/-}$ mice from litters demonstrated to have pLNs (brachial, axillary, mandibular, iliac) and mucosal LNs (mesenteric, cervical, sacral) (Table IV) were assayed in the CH model. All mice were examined at necropsy to confirm which LNs were present. The transient exposure to the anti–LTβ-R mAb during gestation did not alter the lymphoid microenvironment of the spleen of LTα$^{-/-}$ mice, as immunohistochemical examination revealed that these mice still had disrupted MZs, lacked T/B cell segregation, and had no FDCs (data not shown). The results showed that LTα$^{-/-}$ mice having LNs generated a CH response that was comparable to the response of LTα$^{+/+}$ mice, whereas the response of LTα$^{-/-}$ mice without LNs was grossly impaired (Table IV).

LTβ$^{-/-}$ Mice that Lack LNs Cannot Respond to Hapten Challenge. LTα$^{-/-}$ mice and LTβ$^{-/-}$ mice share defects in LN and PP genesis, spleen and LN cellular organization, and humoral responses, but differ in regard to the presence of LTα homotrimers and of mucosa-associated LNs (3). In contrast to LTα$^{-/-}$ mice, LTβ$^{-/-}$ mice responded at the same level as LT$^{+/+}$ or C57Bl/6 control mice to both FITC or oxazalone challenge in the CH model (Table V, and data not shown). Importantly, the LTβ$^{-/-}$ mice used for these studies lacked all LNs except mesenteric, sacral, and cervical, none of which directly drain the sensitized area of the skin. We next investigated the capacity for CH responses in LTβ$^{-/-}$ mice that lacked all LNs.

The original colonies of LTβ$^{-/-}$ mice retained mLNs, cervical LNs, and sacral LNs, which was true regardless of whether the gene deletion was on a pure C57BL/6 or mixed 129/C57BL/6 backgrounds (15, 16; and unpub-

Table VI. *Balb/c Mice which Selectively Lack Peripheral and mLNs Have a Normal CH Response to Hapten whereas Balb/c Mice which Lack All LNs Do Not Respond to Hapten Challenge*

LN	Sensitization	Elicitation	Percent swelling		
			Expt. 1	Expt. 2	Expt. 3
All	FITC	FITC	75 ± 13	62 ± 13	30 ± 6
All	FITC	Vehicle	0 ± 3*	5 ± 2*	nd
All	Vehicle	FITC	nd	nd	3 ± 3*
Mucosal LNs‡	FITC	FITC	51 ± 12	43 ± 19	25 ± 10
mLNs only§	FITC	FITC	nd	nd	33 ± 4
None	FITC	FITC	nd	nd	9 ± 5*

For the CH experiments, the adult progeny were painted with 100 μl FITC then rechallenged 10 d later on the ears with 10 μl FITC. Change in ear thickness was determined. All mice were examined for the presence of LNs at necropsy. Values shown are percent increase in ear thickness (n = 6).
*$P < 0.005$ for values significantly less than FITC control.
‡Pregnant Balb/c mice received LTβ-R-Ig and TNF-R55-Ig fusion proteins, and their progeny developed none or varying numbers of mucosal LNs (see text).
§These mice had 1–3 mLNs only.

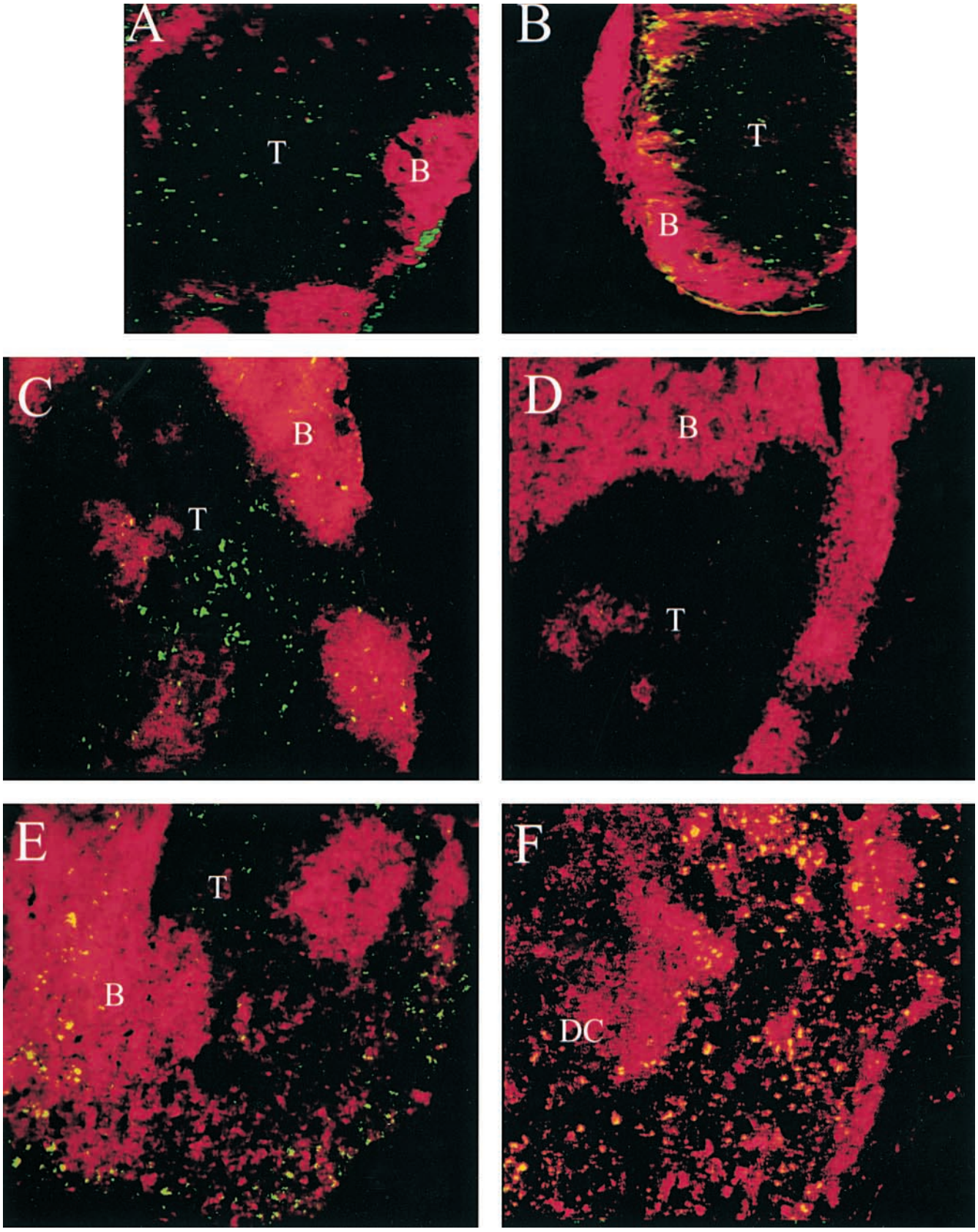

Figure 1. Immunohistochemical characterization of FITC-laden LCs within LNs. (A–E) Immunohistochemical images of LNs taken from mice 24 h after FITC painting: brachial LNs from $LT\alpha^{+/+}$ (A) and $LT\alpha^{-/-}$ mice (B). Original magnification: ×50. FITC reactivity is present in the subcapsular sinus and in the T cell area; counterstaining is PE–anti-B220. mLNs are from Balb/c mice which lack peripheral LNs (C and F), from a Balb/c mouse which has pLNs (D), and from an $LT\beta^{-/-}$ mouse (E). Original magnification: ×100. FITC reactivity is in the T cell area of the Balb/c mLN (C, red

lished data). The presence of various mucosa-associated LNs in $LT\beta^{-/-}$ mice versus their absence in $LT\alpha^{-/-}$ mice has been interpreted as evidence for additional signaling components in the LT system, e.g., via $LT\alpha^{-/-}$ or LIGHT (15). However, a complete lack of mLNs was observed in up to 25% of the $LT\beta^{-/-}$ mice in three separate colonies (15, 16; and unpublished data). Furthermore, considerable variation in the LN content of $LT\alpha^{-/-}$ mice was noted in different colonies with mLNs being present in 5–25% of $LT\alpha^{-/-}$ mice in three separate colonies (13, 14; and unpublished data). Mice lacking mLNs were only rarely observed in the original colony of $LT\beta^{-/-}$ mice (15); however, after sterile rederivation and housing in our specific pathogen-free facility, we now find $LT\beta^{-/-}$ mice which have no LNs (40%), retain only a single mLN or several mLNs and cervical LNs, (40%), or have all mLNs, sacral LNs, and cervical LNs (20%). A similar change in phenotype has been reported in a separately derived colony of $LT\beta^{-/-}$ mice (19). It now appears that LN formation is largely defective in these mice.

To determine if the ability of $LT\beta^{-/-}$ mice to respond to hapten challenge depended on the presence of LNs, $LT\beta^{-/-}$ mice were tested in the FITC or oxazalone CH models then examined at necropsy to determine which LNs were present. In contrast to $LT\beta^{-/-}$ mice that retained LNs, those without LNs failed to mount a CH response to either FITC or oxazalone (Table V, and data not shown). Strikingly, a single remaining mLN was sufficient to generate a CH response. In agreement with the BM transfer and $LT\alpha^{-/-}$ studies, these results suggest that the ability of $LT\beta^{-/-}$ mice to generate a CH response depends on the presence of LNs.

Hapten-mediated CH Responses Are Blocked in Normal Mice that Lack All LNs. As the presence of LNs in $LT\alpha^{-/-}$ mice restored the ability to mount a CH response, we next asked if the loss of LNs from wild-type mice would ablate the CH response. Using either LTβ-R-Ig or a combination of LTβ-R-Ig and TNF-R55-Ig, we can reproducibly block the development of only pLNs, or of peripheral and mucosal LNs, respectively (12). By varying the time at which dosing with antagonists is initiated, the extent of LN development can be manipulated (12). Furthermore, one or two mLNs are occasionally "spared" in some pups treated with LTβ-R-Ig and TNF-R55-Ig starting at day 11 of gestation (12). Using these methods we have engineered normal mice having variable numbers of mLNs, but lacking all other LNs (Table VI). We tested the immunocompetence of these mice in CH models, and evaluated at necropsy which LNs were present.

Mice retaining mucosal LNs but no pLNs were capable of efficient CH responses, whereas mice which lacked all LNs failed to respond to challenge with either FITC or oxazalone (Table VI and data not shown). These results strongly suggested that the ability of individual mice to respond to hapten challenge was due to the presence of LNs.

The minimal LNs required to support a CH response was examined using mice from litters given dual treatment in utero beginning on day 11 of gestation and having progressively fewer peripheral or mucosal LNs. All mice which retained a single LN were capable of responding to hapten challenge whereas those which had no LNs failed to respond to the challenge (Table VI). This was true within litters having some pups with a single mLN and littermates having no LNs. Therefore, even a single nondraining LN was sufficient to enable a CH response.

Trafficking of LCs in $LT^{-/-}$ Mice and in Normal Mice Lacking pLNs. As mentioned earlier, immunohistochemical examination of the skin of wild-type and $LT\alpha^{-/-}$ mice had not revealed any difference in the extent or pattern of LC staining (unpublished data). However, it was critical to demonstrate that LCs could traffic from the skin to LNs in the $LT^{-/-}$ mice, as the increase in LC number isolated after hapten painting correlates with T cell proliferative activity in the LNs (26). Therefore, various $LT^{-/-}$ mice were painted with FITC, and the draining brachial and axillary LNs (if present), the mLNs (if present), and the spleen were harvested 24 h later. Mice painted with vehicle alone had no FITC reactivity in the draining LNs (data not shown). Little or no FITC staining was found in the spleens of painted control, $LT\alpha^{-/-}$, or $LT\beta^{-/-}$ mice (data not shown). FITC reactivity in LNs taken from $LT^{+/+}$, $LT\alpha^{-/-}$, or $LT\beta^{-/-}$ mice 24 h after painting was primarily limited to the T cell areas and adjacent sinus (Fig. 1, and data not shown). The source of FITC reactivity in the LNs of FITC-painted mice was confirmed to be LCs using manual disruption of LNs followed by metrizamide isolation and FACS® analyses. LC purity after isolation was ~70%, with the remainder being primarily B cells and T cells. LCs, defined based on large size and complexity, were $FITC^+$, $CD11c^+$, $NLDC145^+$, MHC class II bright, and B7-2 bright (data not shown). From these data we concluded that the fluorescent staining in LNs detected by immunohistochemistry was due to the influx of LCs carrying FITC, as reported previously (26–31).

These results suggested that LCs could traffic to remaining LNs to trigger T cell activation, and thereby support a CH reaction upon rechallenge. When pLNs were absent, FITC reactivity was readily observed in the T cell areas of the nondraining mLNs (Fig. 1, B–E), whereas little or no FITC reactivity could be found in the white pulp zones of the spleen (data not shown). When draining pLNs were present, FITC reactivity was not observed in nondraining LNs or in mLNs (Fig. 1 D). Therefore, LCs preferentially accumulate in the first LNs they encounter, which is normally the draining LNs, as noted previously (10). When the draining LNs are absent, LCs can home to any LNs. In

staining is PE–anti-B220; and F, red staining is PE–anti-CD11c). FITC-laden cells are also $CD11c^+$, as are resident DCs (F). Trafficking LCs are both $FITC^+$ and $CD11c^+$ and so appear yellow. In the $LT\beta^{-/-}$ mouse, FITC reactivity is present in the T cell area, as well as the disrupted B cell areas and adjacent sinus (E, red staining is PE–anti-B220). T, T cell area; B, B cell area.

contrast, either LCs do not home to splenic white pulp, or the density of LCs that reach the spleen is insufficient to trigger a CH response. To further demonstrate that hapten specific responses were generated in mLNs but not spleen, we performed adoptive transfer experiments. Balb/c mice lacking all pLNs, but retaining mLNs and spleen, were sensitized with oxazalone. 4 d later mLN cells and splenocytes were isolated and transferred to normal, naive Balb/c mice, which were then challenged by ear painting with oxazalone. The CH response was measured after 24 h, and the results (Fig. 2) demonstrate that cells isolated from mLNs, but not spleen, were capable of adoptively transferring the capacity for CH to naive recipients. Therefore LN-specific homing by LCs is essential for the T cell priming underlying the CH response.

Discussion

We have demonstrated that LNs are required to initiate a CH response by using the LT system to manipulate the development of LNs on wild-type and LT-deficient backgrounds. Our critical finding is that CH can occur in $LT\alpha^{-/-}$ mice in which LNs have been induced to develop, regardless of the lack of LTα expression by circulating lymphocytes. LNs were essential for the CH response to haptens which induce cytokines typical of both Th1- (DNFB) and Th2- (FITC and oxazalone) dominated responses (30, 31, and references therein). This observation was consistent in all experimental settings when $LT\alpha^{-/-}$ mice and $LT\beta^{-/-}$ mice with or without LNs were contrasted, and further supported by the BM transplantation studies using $LT\alpha^{-/-}$ mice. These results are fully consistent with the paradigm that LNs are a critical site of T cell activation in response to epidermal antigens.

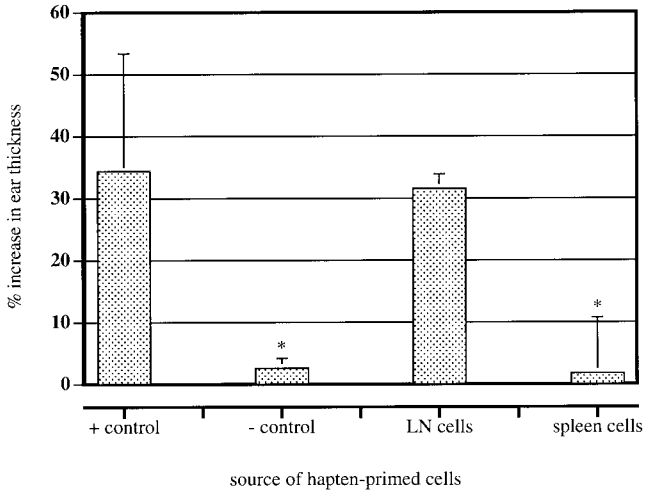

Figure 2. mLN cells, but not splenocytes, can adoptively transfer CH to naive recipients. Balb/c mice having mLNs, but no pLNs, were sensitized with oxazalone. 4 d later cell suspensions of mLN ("LN cells") and spleen ("spleen cells") were harvested and injected intravenously into normal, naive, recipient mice, which were challenged to induce CH. "+ control" refers to mLN^{+}/pLN^{-} mice which were sensitized and challenged with oxazalone. "− control" refers to mLN cells isolated from nonsensitized mice. $^{*}P < 0.001$ for values significantly less than the positive control ($n = 6$).

The earliest modern investigations of CH grew out of efforts to understand the pathology associated with human responses to poison ivy, Japanese lacquer, and other substances which caused contact reactions. Early work focused on identifying the inducing agents, which led to the discovery of a variety of sensitizing compounds. Some investigators of the period hypothesized that contact sensitization was due to the diffusion of antigen or conjugates via epidermal channels, whereas others stressed the importance of passage via lymphatic vessels or the bloodstream (6, and references therein). A functional role for the draining LNs was specifically postulated somewhat later (32), but the anatomy of the sensitization reaction remained controversial for many years, with the mechanistic focus remaining on the site of sensitization, that is, the skin itself (33, and references therein). The relationship between epidermal sensitization and the lymphatic system became clearer as a understanding of lymphocyte and dendritic cell populations developed, particularly with reference to presence of LCs in the epidermis (26, 34). Finally, it was determined that the induction of CH can be blocked by antagonism of various LC properties: activation, migration to LNs, or ability to activate T cells (4, 5, 8, 9, 28, 31). Thus, data accumulated over the course of many decades supported the hypothesis that LCs migrated to LNs, which hosted the T cell–dependent immune response to epidermal antigen, and therefore the development of CH.

We formally tested the hypothesis that LN structure provides a critical environment which supports CH responses by using in utero blockade of the LTβ-R and TNF-R pathways to eliminate LNs from wild-type mice. In utero antagonism of the signaling pathways is transient, and our previous studies have indicated that although the organogenic defects are irreversible, secondary lymphoid architecture, including B cell localization, integrity of splenic MZ populations, and LN addressin expression, is restored in the adult progeny (22, 23). Furthermore, we have recently shown that wild-type mice treated in utero with LTβ-R-Ig are capable of antigen-specific T cell responses (35). Therefore, the simplest model to explain the loss of CH responsiveness in wild-type mice lacking LNs is that LNs provide the proper environment to ensure productive interaction of LCs with circulating T cells. Furthermore, because LCs do not appear to access the splenic white pulp, the spleen is incapable of compensating for the loss of LNs. Finally, any other encounter of LCs with potentially responsive T cells is either very rare or functionally irrelevant (2).

Surprisingly, the CH response can be triggered in LNs that do not directly drain the site of antigen exposure; instead, any LN within the intact lymphatic network is sufficient to support the required LC/T cell interaction. We have demonstrated that antigen-bearing LCs traffic to distant LNs; the route of traffic, however, cannot be determined from these studies. Lymph typically drains into the bloodstream via the thoracic and right subclavian ducts. It

is possible that antigen-bearing LCs are carried by normal circulation to tissue beds and then drain to remaining LNs via afferent lymphatics; however, it is also possible that they are entering LNs directly from the bloodstream by crossing HEVs. Regardless of the route of traffic, the adoptive transfer experiments clearly show that T cell priming to hapten has occurred in the mLNs, but not the spleen, of pLN–deficient mice.

Several recent studies have addressed the role of different secondary lymphoid compartments in mediating immune responsiveness. An essential role for LNs in antiviral immunity was suggested by studies using the LN-deficient alymphoplastic (*aly/aly*) mice. *Aly* mice were unable to control LCMV infections, and BM transfer studies supported the hypothesis that the impaired antiviral response was due to the lack of LNs (36). This hypothesis was supported by the observation that the Hox11$^{-/-}$ mice, which lack the spleen but have LNs, retained a normal antiviral response (36). LT$\beta^{-/-}$ mice also had a defective antiviral responses, with significantly delayed kinetics of lymphocytic choriomeningitis virus (LCMV) clearance (37). The LT$\beta^{-/-}$ mice used in this LCMV study retained mucosal LNs; moreover, it appeared that the LTβ-dependent defect was manifested because it caused disrupted splenic organization, particularly of the MZ. However, this conclusion is at odds with the Hox11$^{-/-}$ mouse study. In addition, some antiviral responses can be manipulated in a manner which may be independent of the status of secondary lymphoid tissue (38). Wild-type mice lacking LNs and/or spleen should provide a useful model to study antiviral responses and address these issues.

Recently, it was demonstrated that an immune response to intradermally administered tetanus toxoid in an immunocompetent SCID-hu model required an engrafted human fetal LNs (39), suggesting the mouse spleen could not trigger an immune response in this setting. Another recent study demonstrated functional overlap of spleen and LN responses, in which transplant rejection could be mediated in the absence of LNs (and PPs), or spleen, but not in the absence of all secondary lymphoid tissue (40). Functional overlap of mLNs and PPs for gut immune responses was also recently demonstrated (35). In contrast, skin transplant rejection required LNs, but not spleen (40), a requirement similar to that described in this study. Finally, the spleen is known to be essential for immunity to encapsulated bacteria (41), and the splenic MZ compartment has recently been shown to be critical for the immune response to T cell–independent antigens (42). These observations suggest that each secondary lymphoid compartment has evolved, at least in part, to respond to specific types of antigen presentation.

Our results invite a reinterpretation of several other studies of immune responsiveness in LT$^{-/-}$ mice. For example, LT$\alpha^{-/-}$ mice and LT$\beta^{-/-}$ mice responded differently to myelin oligodendrocyte glycoprotein (MOG) peptide immunization in a model of experimental autoimmune encephalomyelitis (EAE) development, suggesting that LTα was required for this response (43). However, a second study suggested that genetic background, immune incompetence, and/or altered lymphoid structure in LT$\alpha^{-/-}$ mice accounted for their failure to respond in an EAE model (44). This hypothesis was supported by results demonstrating that robust disease developed upon MOG peptide immunization after transfer of LT$\alpha^{-/-}$ BM cells to recombination activating gene (RAG)$^{-/-}$ recipients. These observations suggest that it is difficult to compare LT$\alpha^{-/-}$ mice, LT$\beta^{-/-}$ mice, or TNF$^{-/-}$ mice in such disease models without taking into account additional variables such as LN status, cellular organization, and other developmental defects, e.g., on hematopoiesis (45). The data presented here support the interpretation that the LT$\alpha^{-/-}$ and LT$\beta^{-/-}$ mice are relatively similar in immune competence, and differences in their disease susceptibility stem from the presence and absence of LNs. Recent work suggesting a critical role for draining LNs in diabetes (46) indicates that there will be similar complexity in interpreting the role of the LT axis, separate from the role of LNs, in several disease models.

The fact that LT ligands did not play a more dominant role in the CH response is perhaps surprising in light of recent data. LT$\alpha^{-/-}$ mice have reduced numbers of resident DCs within secondary lymphoid organs, probably due to reduced expression of chemokines, including secondary lymphoid tissue chemokine (SLC; CCL21) and EBV-induced molecule 1 ligand chemokine (ELC; CCL19) (47–50). This reduction in resident DC number reflects defective traffic into the T cell zones of LNs, rather than the failure of DCs to develop (48). The reduction in resident DC number may be partially offset by the loss of LNs and PPs in LT-deficient or wild-type mice, such that DCs may accumulate in tissues such as spleen or remaining LNs; however, this has not been investigated. Certainly the localization of DCs, and of LCs which reach LNs in LT-deficient mice appears disrupted, being less restricted than normal to the T cell zone.

The concept of "T zone tropism" (1) is well illustrated by the paucity of LN T cells (*plt*) mouse, in which both T cells and DCs fail to migrate into the T cell zone of spleen and mLNs, due to the lack of expression of SLC and ELC in LNs (51, 52). When *plt* mice were challenged on the skin with hapten, 75% fewer LCs accumulated in draining LNs than in wild-type mice (51). Therefore, LCs traffic into LNs is also influenced by expression of these chemokines. However, CH responses in these mice appeared normal, and, although the site of priming was not identified, labeled epidermal DCs could not be found in the spleen (53). It does appear that supraoptimal doses of hapten were used in this study (54), which may complicate interpretation of the CH result. Indeed, knockout of the SLC/ELC receptor CC chemokine receptor (CCR)7 produced somewhat different results, as the CCR7$^{-/-}$ mice had essentially no T cells or DCs in LNs and did not respond to hapten challenge in the FITC CH model (29). SLC was absent and ELC expression was reduced by 75% in the spleen of LT$\alpha^{-/-}$ mice (49), and it may be the continued expression of ELC in LT$^{-/-}$ mice that supports sufficient accumulation of LCs into LNs to support the CH response.

Alternatively, unknown mechanisms may be operating. Given that multiple genes for SLC have been identified, and that there is some evidence for differential expression of SLC and ELC in secondary lymphoid organs, the epidermis, and lymphatics, the roles of SLC and ELC in the traffic of LCs and DCs into secondary lymphoid organs will require additional investigation (52, 55, 56).

It has been demonstrated that LCs form a continuous reticular barrier which has evolved to trap epidermal antigens (57). Our results highlight not only the critical role that LCs play in mediating epidermal immune responses, but also the essential nature of LNs in supporting LC-driven T cell activation. The mechanisms which allow preferential LC traffic to distant LNs, but not splenic white pulp, are not understood, but may involve specific chemokine responsiveness. The reliance on LNs appears to be unique to LCs residing in the epidermal compartment, as our results indicate that subcutaneous hapten injection results in an intact hypersensitivity response in LT$\alpha^{-/-}$ mice (Table II). This suggests that presentation of subepidermal antigen does not rely solely on LNs, possibly because antigen which reaches the dermal layer will encounter additional populations of APCs (58). Our model predicts that dermal APCs have trafficking properties that allow access to other lymphoid compartments such as spleen in addition to LNs. Indeed, such a mechanism was recently proposed to explain the enhanced response to subcutaneously presented antigen in the *plt* mouse, whereby the T cell response shifted from draining LNs to the spleen (53).

Our studies have conclusively identified an essential role for LNs in a T cell–dependent immune response. Furthermore, we have demonstrated that LCs specifically home to LNs, and will traffic until a LN is encountered, bypassing the spleen. This specific trafficking of LCs to LNs comprises a unique and critical component of the secondary lymphoid system, and illustrates the importance of functional anatomy for immune responses. The signals that support LC access to LNs will be a important area for future study, and may yield therapeutic targets to allow highly specific intervention in CH and other disorders.

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