Mice Lacking Expression of the Chemokines CCL21-Ser and CCL19 (*plt* Mice) Demonstrate Delayed but Enhanced T Cell Immune Responses

By Shigeyuki Mori,* Hideki Nakano,* Kentaro Aritomi,*‡ Chrong-Reen Wang,§ Michael D. Gunn,‖ and Terutaka Kakiuchi*

*From the *Department of Immunology, Toho University School of Medicine, Tokyo 143-8540, Japan; the ‡Department of Orthopedic Surgery, Juntendo University School of Medicine, Tokyo 113-8421, Japan; the §Department of Allergology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; and the ‖Division of Cardiology, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710*

Abstract

The paucity of lymph node T cells (*plt*) mutation leads to a loss of CCL21 and CCL19 expression in secondary lymphoid organs. *plt* mice have defects in the migration of naive T cells and activated dendritic cells into the T cell zones of lymphoid organs, suggesting that they would have defects in T cell immune responses. We now demonstrate T cell responses in *plt* mice are delayed but ultimately enhanced. Responses to contact sensitization are decreased at day 2 after priming but increased at day 6. After subcutaneous immunization, antigen-specific T cell proliferation and cytokine production in *plt* mice are increased and remain markedly elevated for at least 8 wk. Compared with wild-type mice, a proportion of T cell response in *plt* mice are shifted to the spleen, and prior splenectomy reduces the T cell response in draining lymph nodes. After immunization of *plt* mice, T cells and dendritic cells colocalize in the superficial cortex of lymph nodes and in splenic bridging channels, but not in T cell zones. These results demonstrate that *plt* mice mount robust T cell responses despite the failure of naive T cells and activated dendritic cells to enter the thymus dependent areas of secondary lymphoid organs.

Key words: chemokines • cell migration • lymphoid tissue • immunomodulators • contact hypersensitivity

Introduction

Within the T cell zones of secondary lymphoid organs, a continuous flow of naive T cells sample antigens presented by dendritic cells (DCs).[1] During an immune response, a small percentage of these T cells will encounter their cognate antigen in the context of MHC and costimulatory signals provided by DCs and cytokines present within the T cell zone (1). This encounter initiates a series of changes in the numbers, activities, and migration patterns of antigen-specific T cells (2). It is believed that chemokines contribute to this process by controlling the localization of various leukocyte subsets within lymphoid organs (3–6). Recent studies have demonstrated the role of chemokines in several leukocyte trafficking events thought to be important in immune response (3, 7). However, the manner in which leukocyte localization contributes to immune response remains largely unknown.

Two constitutively expressed chemokines, CCL21 (formerly secondary lymphoid tissue chemokine [SLC]) and CCL19 (formerly EBI1 ligand chemokine [ELC]), share a common receptor, CC chemokine receptor (CCR)7 (8, 9). CCL21 is expressed in the high endothelial venules (HEVs) of LNs and Peyer's patches (PPs), within the T cell zones of LN, spleen, and PP, and in the lymphatic endothelium of multiple tissues (10). Normal mice posses at least two independent CCL21 genes, *Scya21a* and *Scya21b* (11, 12).

S. Mori and H. Nakano contributed equally to this work.

H. Nakano's present address is Division of Cardiology, Department of Medicine, Duke University Medical Center, Durham, NC 27710.

C.R. Wang's present address is Department of Internal Medicine, National Cheng Kung University Hospital, 138 Sheng Li Rd., Tainan 700, Taiwan.

Address correspondence to H. Nakano, Division of Cardiology, Department of Medicine, Duke University Medical Center, Box 3623, Durham, NC 27710. Phone: 919-668-0334; Fax: 919-684-8591; E-mail: nakano@duke.edu

[1]*Abbreviations used in this paper:* BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; CMFDA, 5-chloromethylfluorescein diacetate; DC, dendritic cell; HEV, high endothelial venule; PCl, picryl chloride; *plt*, paucity of LN T cells; PP, Peyer's patch; WT, wild-type.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2001/01/207/11 $5.00
Volume 193, Number 2, January 15, 2001 207–217
http://www.jem.org/cgi/content/full/193/2/207

Scya21a encodes a serine at position 65 of CCL21 (CCL21-Ser) and is expressed in both secondary lymphoid organs and lymphatics. The *CCL21b* gene, encoding leucine at position 65 (CCL21-Leu), is expressed only in the lymphatic endothelium of peripheral tissues. CCL19 is expressed predominately by stromal cells within the T cell zones of LN, spleen, and PP (13, 14). The receptor for these ligands, CCR7, is expressed on all naive T cells, some memory T cells, B cells, and activated DCs (15–18). Receptor occupancy by either ligand stimulates chemotaxis and activation of leukocyte integrins (10, 19, 20).

We identified a spontaneous mutation in mice characterized by a defect in the homing of naive T cells to LNs, PPs, and splenic white pulp (21, 22). This mutation, paucity of LN T cells (*plt*), involves a genomic deletion that includes the CCL21 gene expressed in lymphoid organs (*Scya21a*) and the CCL19 gene (*Scya19*; references 11, 12, and 14). *plt* mice do not express CCL21 or CCL19 protein in secondary lymphoid organs but continue to express CCL21 at reduced levels in lymphatic endothelium. *plt* mice have three recognized defects in leukocyte localization. First, naive T cells do not adhere to LN or PP HEVs of *plt* mice and therefore display a near total loss of extravasation at these sites (23, 24). This defect is thought to be due to a requirement for CCL21 in the activation of lymphocyte integrins and can be partially reversed by treatment with exogenous CCL21 (23). Naive T cell entry into splenic white pulp is similarly affected. Second, *plt* mice demonstrate a marked paucity of DCs within T cell zones and a decreased migration of activated DCs to the T cell zones of spleen and LNs (25). Expression of CCR7 is induced in DCs upon activation, suggesting that CCL21 and/or CCL19 is involved in the migration of these cells to T cell zones (15, 16). Third, because CCL21 appears to contribute to the maintenance of distinct T and B cell zones (26), *plt* mice demonstrate a lack of recognizable T cell zones and an increased number of T cells in and around lymphoid follicles (22).

Like *plt* mice, mice lacking CCR7 demonstrate disrupted homing of naive T cells to T cell zones, decreased migration of activated DCs from skin to LNs, and an abnormal distribution of T cells within secondary lymphoid organs (27). The DC migration defect appears to be more severe in $CCR7^{-/-}$ mice than in *plt* mice, suggesting that lymphatic expression of CCL21 is required for the migration of DC into lymphatics. $CCR7^{-/-}$ mice demonstrate delayed humoral responses to T cell–dependent antigens. In models of contact hypersensitivity and delayed-type hypersensitivity, it has been reported that $CCR7^{-/-}$ mice lack primary T cell responses (27).

In our previous report, we observed that *plt* mice are profoundly sensitive to infection with murine hepatitis virus (25). This finding, taken with the reported lack of T cell responses in $CCR7^{-/-}$ mice, strongly suggested that *plt* mice would also be unable to develop primary T cell responses. In contrast to this prediction, we now demonstrate that *plt* mice are capable of mounting robust T cell responses. The responses of *plt* mice to contact sensitization and subcutaneous immunization are delayed, but ultimately enhanced compared with that seen in wild-type (WT) mice. More strikingly, Ag-specific T cell responses in the LNs and spleens of *plt* mice do not decline after reaching peak levels, suggesting that the activation of T cells within the thymus dependent areas of secondary lymphoid organs is required for the contraction phase of immune response.

Materials and Methods

Mice and Immunization. BALB/c mice were purchased from Shizuoka Laboratory Corporation and bred in the animal facilities in Toho University School of Medicine. BALB/c-*plt/plt* mice were produced by back crossing DDD/1-*plt/plt* mice to BALB/c 10 times and bred in the facilities. Mice were used at 8 to 12 wk of age. They were immunized subcutaneously with 100 μg of chicken OVA (Sigma-Aldrich) in a 1:1 emulsion of PBS and CFA (Difco) in the footpads or at the base of the tail. In some experiments, spleens were surgically removed from mice at least 9 d before immunization.

Contact Hypersensitivity Response. Mice were sensitized epicutaneously with 150 μl of 7% 2,4,5-trititrochlorobenzene (picryl chloride [PCl]; Nacalai Tesque) in acetone/olive oil (4:1) on their shaved abdomens. 2 or 6 d after sensitization, their right ears were challenged with 20 μl 1% PCl in vehicle. As a control, their left ears were challenged with vehicle alone. At various times after challenge, ear thickness was measured with a dial thickness gauge (Peacock).

In Vitro Recall Response of T Cells. Single cell suspensions were prepared from draining popliteal and inguinal LNs or spleens and pooled from 3–4 mice at 2–56 d after immunization. Cells suspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS and 5×10^{-5} M 2-ME were incubated with the indicated concentrations with OVA in 96-well culture plates in humidified atmosphere of 5% CO_2 in air at 37°C for 4 d. The cell proliferation was assessed by incorporation of [^{3}H]TdR (Amersham Pharmacia Biotech), which was determined on a Matrix 96 direct beta counter (Packard Instrument Co.) and the results were expressed as counts/3 min. To assess IL-2 production, 50 μl of culture supernatants were harvested at 24 h of culture, and IL-2 activity was determined using the IL-2–dependent T cell line, CTLL-2. Proliferation of CTLL-2 was assessed as described above. In some experiments, T cells were enriched from LN cells by the passage through nylon wool column followed by the treatment with anti–mouse IgM rabbit IgG and anti–MHC class II mAb M5/114, and rabbit complement. The purity of $CD3^+$ T cells was >95%. Enriched T cells were incubated with OVA in the presence of 33 Gy x-ray–irradiated BALB/c spleen cells (5×10^5/well).

Flow Cytometric Analysis. To analyze lymphocyte subpopulations, cells were stained with appropriate mAbs conjugated with FITC, PE, or biotin. Cells treated with biotinylated mAb were further treated with PE-conjugated streptavidin. Anti–TCR-α/β (H57-597), anti-CD8 (53-6-7.2), anti-B220 (RA3-3A1/6.1), anti–I-A^d (MK-D6), and anti-CD11c (N418) mAbs were used. Stained cells were analyzed on Cytron Absolute (Ortho Diagnostics) or FACSCalibur™ (Becton Dickinson) flow cytometers.

Histological Analysis. 9 d after immunization, draining popliteal and inguinal LNs and spleen were harvested and frozen. Cryostat sections (6 μm), preincubated with PBS containing 2% rabbit serum and 1% BSA, were stained with FITC-conjugated anti-Thy1.2 mAb (30H12), FITC-conjugated or biotinylated anti-B220 mAb (RA3-3A1/6.1), or anti-DEC205 (NLDC-145) followed by biotinylated rabbit anti–rat IgG (Jackson Immu-

noResearch Laboratories). For fluorescent analysis, biotinylated Abs were followed by streptavidin-conjugated Cy3 (Jackson ImmunoResearch Laboratories), and sections were analyzed on an MRC-600 confocal laser microscope system (Bio-Rad Laboratories). For immunohistochemical analysis, sections were stained with horseradish peroxidase–conjugated rabbit anti-FITC Abs (Dako) and streptavidin-conjugated alkaline phosphatase, developed with diaminobenzidine and VectorBlue (Vector Laboratories).

In Vivo Migration Assay. Resting lymphocytes were collected from peripheral LNs, mesenteric LNs, and spleens of BALB/c mice. Draining LN cells were collected from draining popliteal and inguinal LNs of OVA/CFA-immunized BALB/c mice at day 9 after immunization. T cells were enriched by panning in goat anti–mouse IgG (Cappel)-coated dishes followed by anti–MHC class II (M5/114) and rabbit complement. T cell–enriched preparation always contained >90% $CD3^+$ cells. To purify activated splenic T cells, splenocytes from BALB/c mice immunized with OVA/CFA were sorted using MACS column (Miltenyi Biotec) with anti-B220 (RA3-6B2), anti–I-A^d (39-10-8), anti-GR1 (RB6-8C5), and anti-CD45RB (16A) Abs (BD PharMingen). Over 99% of enriched splenic T cells were $CD44^{high}$ and 75% were $CD62L^{low}$. 10^6 or 1.4×10^6 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM (Dojindo)– or 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probe)-loaded T cells were injected by tail vein or subcutaneously into a foot pad of untreated or OVA/CFA immunized mice at day 9 after immunization. Mice were killed 6 h after injection, and BCECF- or CMFDA-positive cells in draining (popliteal and inguinal) LNs and nondraining (cervical, axillary, and brachial) LNs were quantitated by flow cytometry.

Results

Contact Hypersensitivity Response in plt/plt Mice. WT and *plt* mice were sensitized with PCl by abdominal skin painting, and ear swelling was assessed upon rechallenge with antigen. When rechallenged 2 d after sensitization, *plt* mice demonstrate a reduced degree of ear swelling at 24 h compared with that of WT mice (Fig. 1 A). However, in contrast to what is normally seen in this assay, ear swelling in *plt* mice increases rather than decreases over subsequent days, such that it is greater than that seen in WT mice by day 3 after rechallenge. When rechallenged 6 d after the sensitizing dose, *plt* mice demonstrate an enhanced response compared with WT mice at all time points examined. This response is rapid, and ear swelling decreases over subsequent days. This finding suggests that T cell priming is intact in *plt* mice although it is delayed relative to WT mice.

Changes in Lymphocyte and DC Numbers after Immunization. *plt* mice have a defect in the migration of naive T cells and DCs to the T cell zones of LNs and spleen that would seem to preclude effective T cell priming (21, 22, 25). The response of *plt* mice to contact sensitization suggests that this defect may be overcome after antigen challenge. To examine this possibility, we immunized WT and *plt* mice subcutaneously with OVA in CFA and examined the numbers of total T cells, $CD4^+$ cells, $CD8^+$ cells, B cells, and DCs within draining LNs and spleen over subsequent days (Fig. 1, B and C). In WT mice, the number of T cells increases rapidly in LNs after immunization (Fig. 1 B). The number of T cells peaks at day 9 and begins to decline by day 20. Consistent with previous findings, the number of $CD4^+$ and $CD8^+$ T cells is markedly reduced in *plt* LNs before immunization. *plt* mice demonstrate almost no increase in the number of LN T or B cells 4 d after immunization. However, by day 9 the number of these cells has increased markedly over baseline levels. T cell

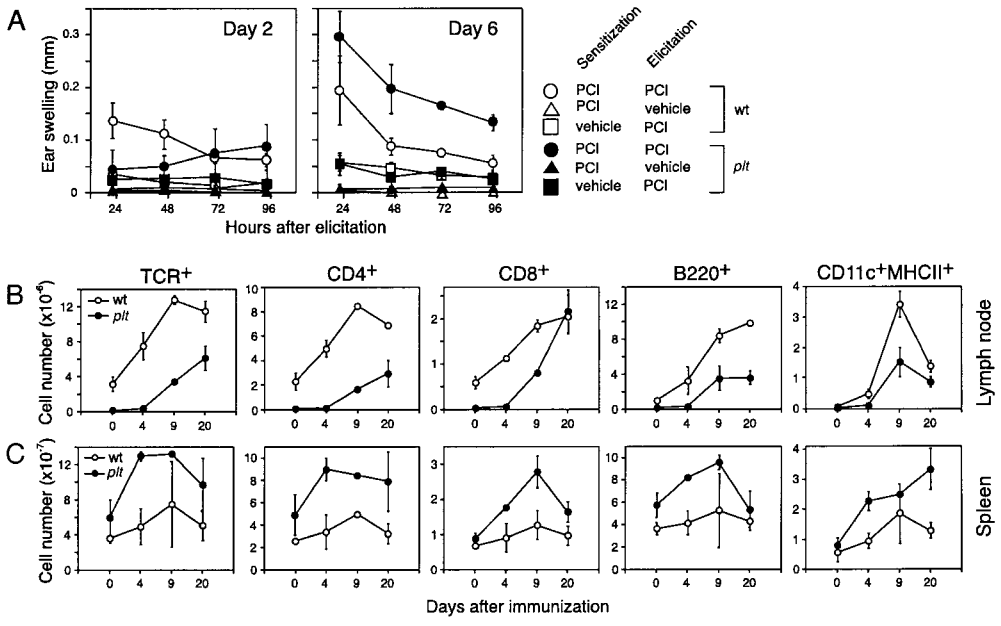
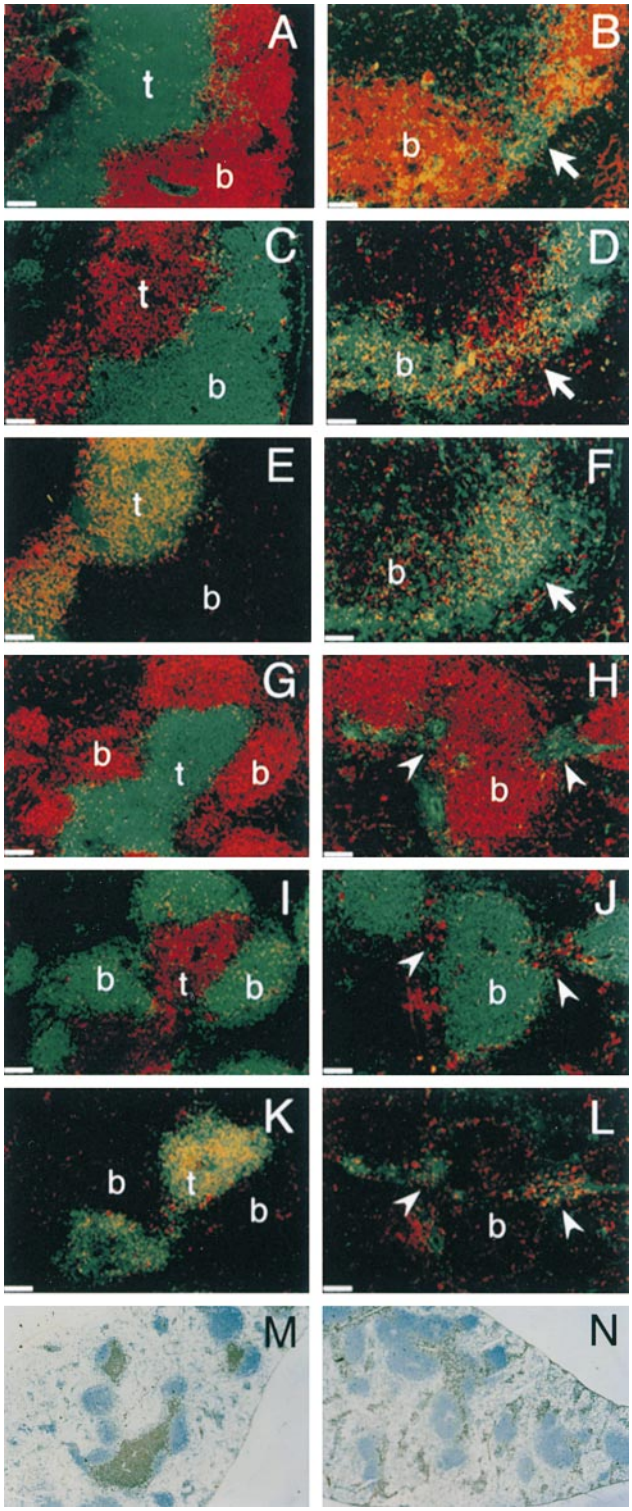


Figure 1. Contact hypersensitivity response and kinetics of leukocyte accumulation in LNs and spleen after immunization. (A) Mice were sensitized with 150 μl 7% PCl in acetone/olive oil or vehicle alone, and 2 or 6 d later, 20 μl of 1% PCl was applied to the right ear with vehicle alone applied to the left ear. Ear thickness was measured at the indicated times after elicitation. Ear swelling was calculated in comparison to baseline. Mean ± SD for four to five mice are indicated. (B and C) Cells from draining LNs (B) or spleen (C) were harvested from WT (○) or *plt* (●) mice at day 0, 4, 9, and 20 after immunization. Cells were counted, stained with anti–TCR-Cβ, anti-CD4, anti-CD8, anti-B220, anti–I-A, or anti-CD11c mAb, and analyzed by flow cytometry. Cell numbers were calculated from the total cell number and the percentage of positive cells, and indicated as the number per single LN or spleen. Mean ± SD for two mice are indicated. Similar results were obtained in a repeat experiment.

numbers further increase by day 20, with the number of $CD8^+$ cells reaching a level comparable to that seen in WT mice. Interestingly, the accumulation of B cells in *plt* LNs is also reduced in the first 4 d after immunization. The accumulation of DCs in *plt* LNs is less than half of that seen in WT mice but occurs over a similar time course.



When spleens are examined, WT mice demonstrate little increase in the number of splenic T cells at 4 d after immunization. By day 9 there is a modest increase in T cell numbers, which falls off by day 20. As shown previously, the spleens of *plt* mice contain an increased number of T cells at baseline (Fig. 1 C). After immunization, T cell numbers increase rapidly in the spleens of *plt* mice, with a twofold increase by day 4 after immunization. These levels remain elevated through day 9 and return towards baseline by day 20. At baseline, the spleens of *plt* and WT mice contain a similar number of DCs. After immunization, DCs gradually accumulate in the spleens of WT mice, with a peak increase of fourfold seen at day 9. In *plt* mice, splenic DCs accumulate more rapidly and to a greater extent than is seen in WT mice.

Localization of T Cells and DCs after Immunization. Because CCL21 and CCL19 are believed to contribute to the proper localization of lymphocytes and DCs within secondary lymphoid organs, we examined the localization of these cells after immunization. Frozen LN and spleen sections were stained with anti-Thy1.2, anti-B220, and anti–DEC-205 9 d after immunization and examined by confocal microscopy (Fig. 2). In WT LNs, T cells and B cells are well segregated into T cell zones and follicles with the majority of colocalization occurring at the intersection of these areas (Fig. 2 A). In *plt* LNs, the majority of T cells are found in the region just beneath the subcapsular sinus and an increased number of these cells is seen within follicles (Fig. 2 B). In WT LNs, the vast majority of DCs are found within the T cell zone (Fig. 2 C) and can be seen to colocalize with T cells in this area (Fig. 2 E). In *plt* LNs, most DCs are found in the superficial cortex between lymphoid follicles (Fig. 2 D) and colocalize with T cells in this area (Fig. 2 F). This region appears to be enlarged compared with WT mice.

As described previously, *plt* mice demonstrate several abnormalities in splenic architecture (22). Total splenic T cell numbers are increased 1.5–3-fold, probably due to the exclusion of these cells from LNs. *plt* mice demonstrate markedly decreased numbers of T cells within white pulp, with a redistribution of these cells into red pulp and into gaps in the marginal zone between red and white pulp known as bridging channels (28, 29). *plt* mice also demonstrate an increased number of white pulp areas, each of these being

Figure 2. Localization of T cells, B cells, and DCs in the draining LNs and spleen of OVA-immunized mice. Draining popliteal LNs from WT (A, C, and E) and *plt* mice (B, D, and F), and spleens from WT (G, I, K, and M) and *plt* mice (H, J, L, and N) were harvested at 9 d after immunization. Frozen sections were stained with mAbs anti-Thy1.2 for T cells (green in A, B, E, F, G, H, K, and L, or brown in M and N), anti-B220 for B cells (red in A, B, G, and H, green in C, D, I, and J, or blue in M and N), and anti–DEC-205 for DCs (red in E, F, K, and L). T and B cells (A, B, G, H, M, and N), B cells and DCs (C, D, I, and J), and T cells and DCs (E, F, K, and L) are shown. t, T cell zones; b, B cell follicles. Arrows indicate the subcapsular sinus. Arrowheads indicate splenic bridging channels. Colocalization signal is indicated as yellow/orange. Bars, 100 μm (A–L). Original magnification: (M and N) ×20.

much smaller than normal (22). This diffuse overall distribution of T cells within *plt* relative to WT spleens is not altered after immunization (Fig. 2, M and N), and the concentration of these cells remains decreased in any given area of *plt* spleen despite their increased total numbers. When the spleens of immunized WT mice are examined by confocal microscopy, both T cells and DCs are found almost exclusively within T cell zones (Fig. 2, G and I) and display a strong colocalization signal in this area (Fig. 2 K). In *plt* mice, few T cells or DCs are detected in the white pulp (Fig. 2, H and J). Instead, these cells are concentrated in bridging channels where they can be seen to colocalize (Fig. 2 L). Thus, although T cells and DCs each demonstrate migration defects in *plt* mice, these defects do not prevent T cells and DCs from colocalizing after immunization. However, this colocalization appears to occur at abnormal locations in *plt* mice.

The Primary T Cell Response in plt/plt Mice. The response of *plt* mice to contact sensitization and the colocalization of T cells and DCs after immunization suggests that at least some T cell priming occurs in *plt* mice. To examine T cell priming directly, we immunized WT and *plt* mice subcutaneously with OVA in CFA and analyzed the recall response of draining LN and splenic T cells upon antigen restimulation in vitro. 9 d after immunization, draining LN T cells from *plt* mice respond well to OVA presented by irradiated WT spleen cells. Unexpectedly, both proliferation and IL-2 production in T cells from *plt* mice are greater than in those from WT mice (Fig. 3 A). These T cell responses in *plt* and WT mice are OVA dose dependent (Fig. 3 B). Examination of the curves for T cell proliferation per number of input cells (Fig. 3 A) reveals a shift to the left in those of *plt* mice. This shift suggests that LN preparations from *plt* mice contain a higher percentage of OVA-reactive T cells than those of WT mice. This higher percentage may be due to the decreased total number of T cells in *plt* LNs (see below) but indicates that T cells in the draining LNs of *plt* mice are well primed by OVA immunization. This finding is consistent with the results seen after contact sensitization. Because antigen presentation appears to be intact in *plt* mice, in subsequent experiments we performed in vitro stimulation using LN cells instead of enriched T cells added to irradiated syngeneic spleen cells. Comparable results were obtained using this method (Fig. 3 C).

To examine the time course of T cell priming in *plt* mice, we immunized *plt* and WT mice with OVA in CFA, harvested draining LN and spleen cells at various time points after immunization, and measured cell proliferation and IL-2 production after in vitro restimulation with OVA. The proliferative response of and IL-2 production by LN cells from WT mice gradually increase between day 2 and 4 after immunization, peak at day 9, and are substantially diminished by day 20 (Fig. 4 A). LN cells from *plt* mice demonstrated a response similar to that of WT cells through day 9 after immunization. However, this proliferative response continues to increase until, 20 d after immunization, it is greater than that seen at any time in LN cells from WT mice. This level of proliferative response is maintained for at least 8 wk after immunization. In vitro IL-2 production by *plt* draining LN cells follows a time course similar to that of proliferation and is also maintained at high levels for at least 8 wk.

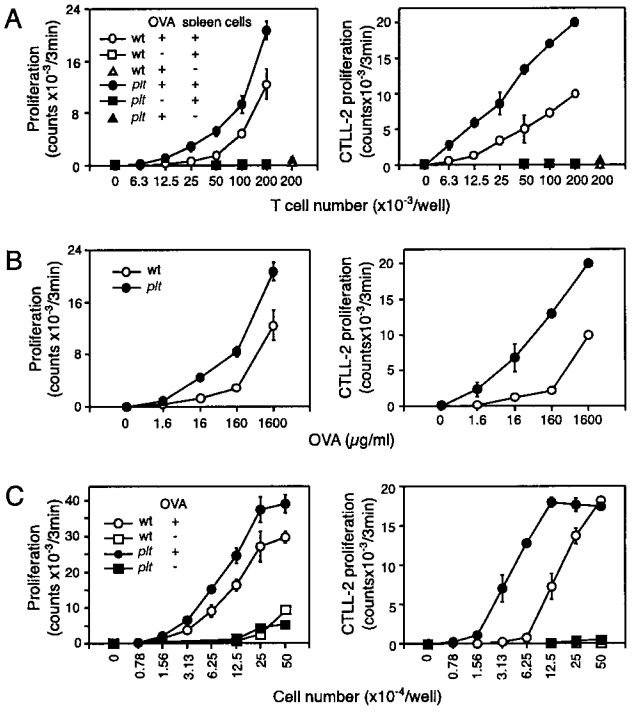


Figure 3. Draining LN T cell responses after OVA immunization. Mice were immunized subcutaneously with 100 μg OVA in CFA. 9 d later, T cells purified from pooled draining LNs or pooled draining LN cells were examined for proliferation and IL-2 production. (A) Sequentially diluted purified draining LN T cells cultured in the presence or absence of 1,600 μg/ml OVA and 5×10^5 of irradiated BALB/c spleen cells. (B) 2×10^5 purified T cells cultured with indicated dose of OVA and 5×10^5 of irradiated spleen cells. (C) Sequentially diluted draining LN cells (unpurified) were cultured with or without 1,600 μg/ml of OVA. Proliferation and IL-2 production were assessed as described in Materials and Methods. Values represent means ± SD from triplicate samples. One of two similar experiments is shown.

When the response of splenic T cells are examined, proliferation of and cytokine production by T cells from *plt* mice is greater than that seen in WT mice at every time point examined (Fig. 4 B). In WT mice, the splenic response to subcutaneous immunization is modest. IL-2 production peaks at day 9 and has begun to decline by day 20. In *plt* mice, the proliferative response develops over the same time course as in WT mice but is greater at all time points. IL-2 production by *plt* splenocytes reaches much higher levels than occurs in WT mice and peaks at day 20.

The above results present T cell responsiveness to in vitro stimulation using a constant number of input cells. However, as shown in Fig. 1 B, the number of T cells in WT and *plt* lymphoid organs differ markedly. To better compare the magnitude of T cell responses in the lymphoid organs of *plt* and WT mice, T cell proliferative responses were normalized to a per draining LN or per spleen basis (Fig. 5). In the draining LNs of WT mice, the total T cell proliferative response increases rapidly until day 9 after im-

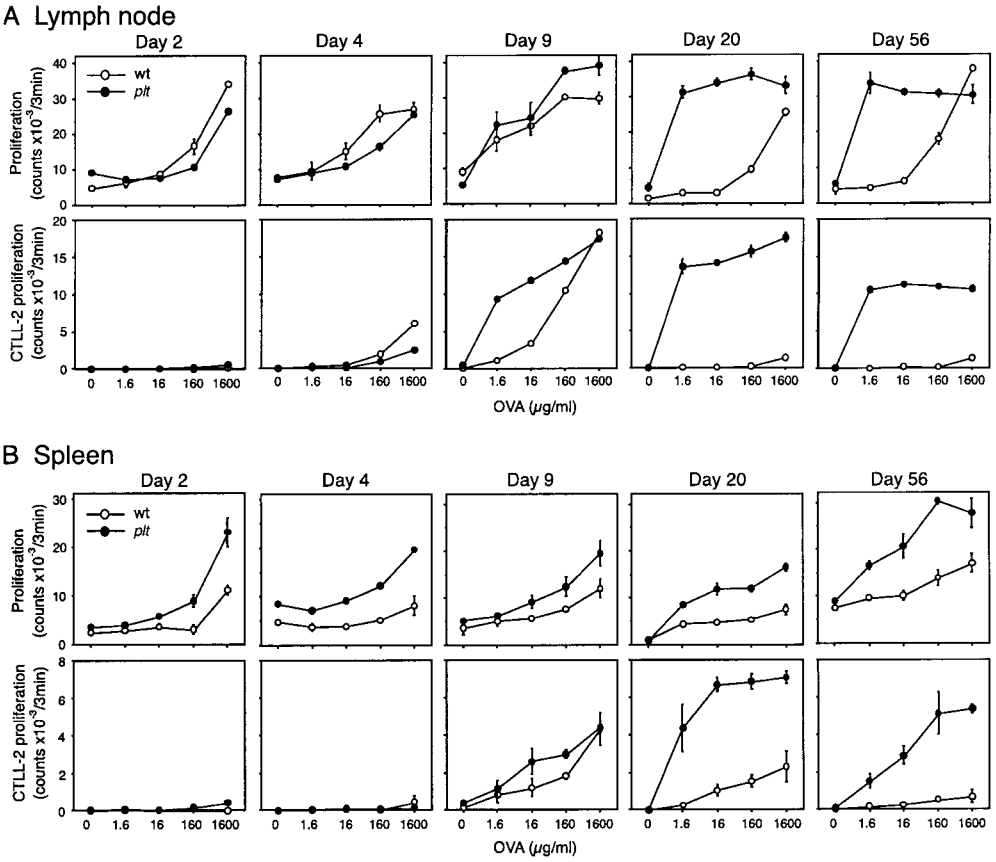


Figure 4. Time course of draining LN and splenic T cell responses after OVA immunization. Draining LN cells (A) or spleen cells (B) were harvested 2, 4, 9, 20, and 56 d after immunization and cultured at 5×10^5 cells/well with various doses of OVA. Proliferation and IL-2 production were assessed as described in Materials and Methods. Means ± SD from triplicate tests are indicated. Representative results from three independent experiments is shown.

munization, then quickly returns to near baseline levels (Fig. 5 A). In the draining LNs of *plt* mice, the total T cell proliferative response increases more slowly, peaks at day 20 at a level ~60% of that seen in WT mice, then decreases at a much slower rate than occurs in WT mice. In the spleens of WT mice, the total T cell proliferative response increases modestly after immunization, then declines slowly (Fig. 5 B). In comparison, *plt* mice mount a much greater splenic T cell response at all time points. This response peaks at day 20 and demonstrates no decrease through 8 wk.

To approximate the total lymphoid organ T cell response, we summed the total T cell proliferative responses from spleen and the four major draining LNs for both WT and *plt* mice. As shown in Fig. 5 C, the total lymphoid organ T cell responses in WT and *plt* mice are surprisingly similar in the first 9 d after immunization. However, between days 9 and 20 the total T cell response in *plt* mice increases dramatically, whereas the response in WT mice declines. Thereafter, the total T cell proliferative responses in WT and *plt* mice decline at a similar rate. This leaves the total T cell response in *plt* mice markedly elevated relative to WT mice.

Primary T Cell Response in the Draining LNs of Splenectomized Mice. The delayed LN response in *plt* mice can be explained by the decreased ability of naive T cells to enter the LNs of these animals from the blood. However, this raises questions about the origin of those T cells that eventually accumulate in the draining LNs of *plt* mice. One possibility is that T cells enter *plt* LNs after being activated at other sites. As the above findings demonstrate, a predominant site of early T cell activation in *plt* mice is the spleen. To evaluate the contribution of splenic activity to the draining LN response of *plt* mice, we immunized WT and *plt* mice that had received prior splenectomy and measured the response of draining LN cells to subsequent in vitro stimulation. When examined 9 and 20 d after subcutaneous immunization, prior splenectomy had little effect on the proliferation of and cytokine production by lymphocytes from WT mice (Fig. 6). However, in *plt* mice T cell proliferation and cytokine production upon in vitro restimulation were substantially reduced in those animals that had received prior splenectomy. These findings suggest that an increased proportion of T cells are primed in the spleens of *plt* mice after immunization and these cells then migrate to draining LNs as the immune response progresses.

T Cell Migration into Draining LNs. T cells entering the draining LNs of immunized *plt* mice may reach this site by two possible routes. First, they may extravasate from the blood across HEVs. Significant extravasation across HEVs would imply that chemoattractants or chemokines other than CCL21 are induced on high endothelial cells during an immune response. Second, T cells may enter draining LNs via afferent lymphatics. This would suggest that only those T cells capable of entering peripheral tissues would reach draining LNs. To distinguish these possibilities, we examined the migration of T cells from blood and subcuta-

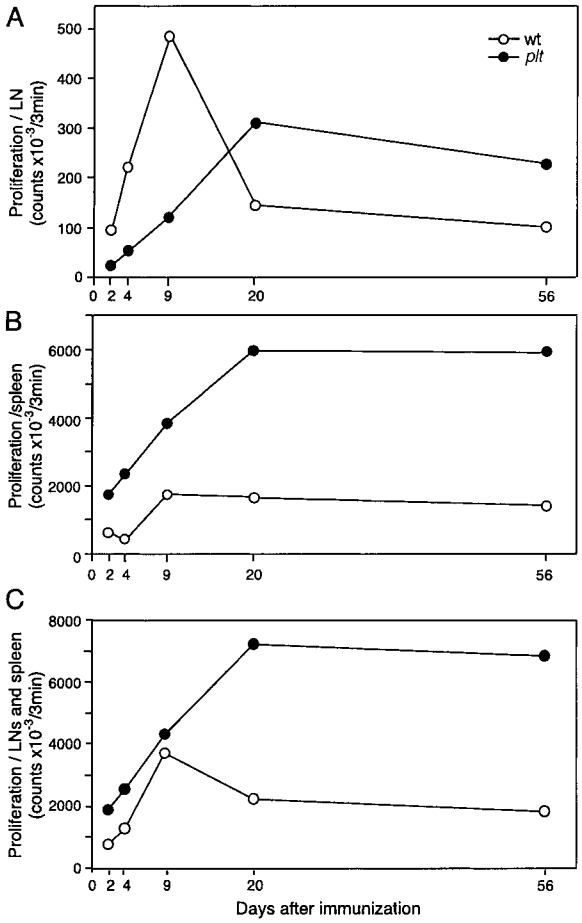


Figure 5. Time course of total draining LN, total splenic, and total lymphoid organ T cell proliferative responses after OVA immunization. Results calculated on the basis of data from Fig. 4. Total proliferative response per draining LN (A) or per spleen (B) was calculated by multiplying the proliferation per cell at 1.6 μg/ml of OVA (derived from Fig. 4) by the mean LN or spleen cell number at the indicated times after immunization. (C) The total lymphoid organ T cell proliferative response was calculated by summing the responses of four draining LNs (two popliteal and two inguinal LNs) and spleen.

neous tissues into the LNs of mice 9 d after immunization. As shown in Fig. 7, A and B, both resting T cells and T cells prepared from the draining LNs of immunized mice migrate well to the LNs of immunized WT recipients with preferential accumulation in draining versus nondraining LNs. Activated T cells purified from the spleens of immunized mice ($CD45RB^-$) show a similar pattern of migration, albeit with a reduced efficiency (Fig. 7 C). When injected into immunized *plt* mice, the migration of all T cell populations to LNs is markedly reduced relative to WT mice (Fig. 7, A–C). This finding is similar to results obtained in nonimmunized *plt* mice (22) and suggests that no other factors compensate for the absence of CCL21 on HEVs after immunization.

To examine the migration of T cells to LNs via lymphatics, cells were injected into footpads and their accumulation in popliteal and inguinal LNs was examined after 6 h. When resting T cells are injected into the footpads of nonimmunized mice, few of these cells reach the draining LNs in either WT or *plt* mice (Fig. 7 D). In contrast, substantial numbers of subcutaneously injected T cells reach the draining LNs of immunized WT mice (Fig. 7, E and F). In *plt* mice, this migration is somewhat decreased in the case of resting T cells but not significantly reduced when draining LN T cells are injected. These findings demonstrate that the lymphatic migration of T cells is preserved in *plt* mice and suggest that this is the major route by which T cells reach the draining LNs of immunized *plt* mice.

Discussion

In a typical primary immune response, presentation of antigen to naive T cells occurs predominantly within the specialized microenvironment of the T cell–dependent areas of secondary lymphoid organs. Although the functional significance of this anatomic specialization is not known, it has often been assumed that T cell priming within T cell zones is required for the proper development of T cell immunity. This view has been supported by the lack of contact sensitization responses in both L-selectin– and CCR7-deficient mice, defects attributed to the severely impaired migration of naive T cells (and in the case of $CCR7^{-/-}$

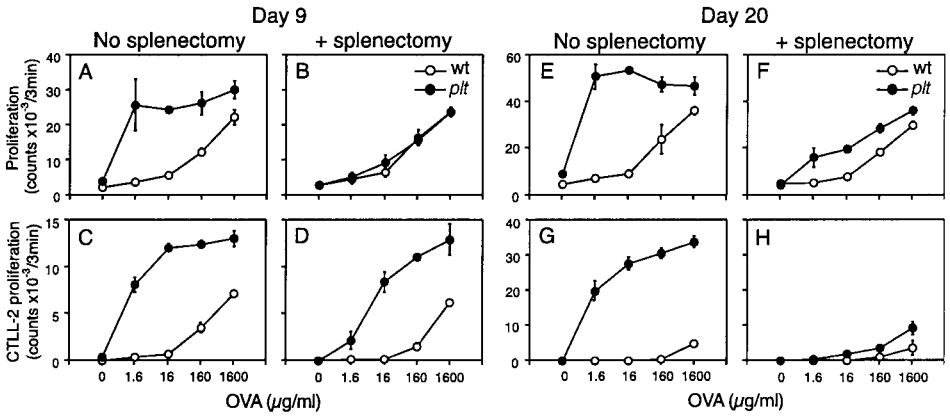


Figure 6. Effect of prior splenectomy on draining LN T cell responses. Spleens were removed from WT (○) and *plt* mice (●) at least 9 d before immunization. Splenectomized and normal mice were immunized subcutaneously with 100 μg OVA in CFA. Draining LN cells were harvested from mice at day 9 (A–D) or day 20 (E–H) after immunization and their proliferation and IL-2 production were measured as in the legend to Fig. 4. Means ± SD from triplicate tests are indicated.

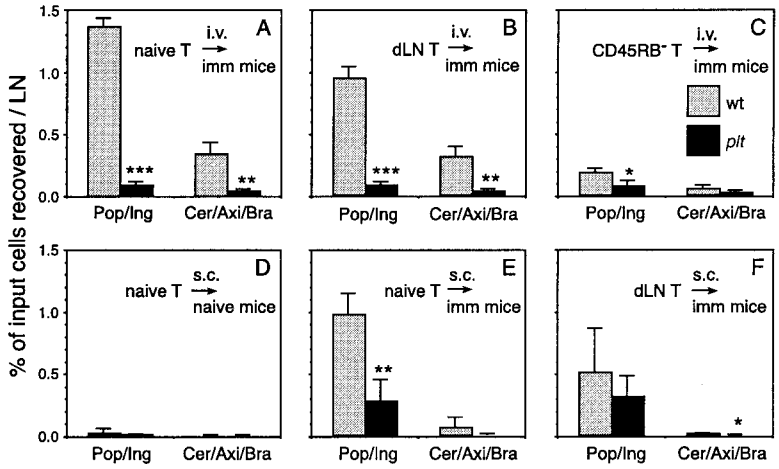


Figure 7. T cell migration into LNs through HEV vs. afferent lymphatics. T cells from nonimmunized mice (naive T), draining LN T cells from OVA-immunized mice (dLN T), or activated ($CD45RB^-$) splenic T cells from OVA-immunized mice ($CD45RB^-$ T) were enriched, labeled with BCECF or CMFDA, and injected into WT (cross-hatched bars) or *plt* (black bars) recipients. All panels show the percentage of injected cells recovered from the draining or nondraining LNs of recipients. (A) 10^7 naive T cells injected by tail vein (i.v.) into immunized (imm) mice. (B) 1.4×10^7 draining LN T cells injected by tail vein into immunized mice. (C) 10^7 $CD45RB^-$ splenic T cells injected by tail vein into immunized mice. (D) 10^7 naive T cells injected into the foot pads (s.c.) of nonimmunized mice. (E) 10^7 naive T cells injected into the foot pads of immunized mice. (F) 10^7 draining LN T cells injected into the foot pads of immunized mice. All immunized mice were used at day 9 after immunization. 6 h after injection of labeled T cells, draining and nondraining LNs were collected and BCECF- or CMFDA-positive cells analyzed by flow cytometry. Percentage of input cells recovered per LN was calculated from total cell count and percentage of the fluorescence-positive cells, and mean ± SD from three mice are indicated. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for *plt* vs. WT. Pop/Ing, popliteal/inguinal; Cer/Axi/Bra, cervical/axillary/brachial.

mice, activated DCs) into the T cell zones of these animals (27, 30, 31). Like $CCR7^{-/-}$ mice, *plt* mice demonstrate impaired migration of naive T cells and activated DCs into T cell zones (21, 22, 25), and therefore could be expected to lack T cell responses.

In contrast to this expectation, we find that T cell priming after antigen challenge is intact in *plt* mice. This demonstrated by two findings. First, *plt* mice respond well to contact sensitization (Fig. 1). Second, T cells from the draining LNs and spleens of immunized *plt* mice demonstrate robust antigen-specific proliferation and IL-2 production after in vitro stimulation (Figs. 2 and 3). When antigen-specific T cell proliferation is normalized to account for differing cell numbers, the overall magnitude of T cell response in *plt* mice equals or exceeds that seen in WT mice at every time point examined. These findings demonstrate that the abnormal immune responses seen in *plt* mice are not due to a failure of T cell priming.

Although T cell immune responses are intact in *plt* mice, they differ from those seen in WT mice in several respects. First, as shown by their response to contact sensitization, T cell responses in at least some peripheral site of *plt* mice are delayed. Second, a portion of T cell responses, especially at early time points, appear to be shifted from LNs to spleen. This shift is seen in the accumulation of lymphocytes and DCs in LNs and spleen after immunization, in the total proliferative response to immunization in LNs and spleen, and in the decreased proliferative capacity of T cells recovered from the draining LNs of *plt* mice that have received prior splenectomy (Fig. 1, B and C, and Figs. 5 and 6). Third, and most striking, T cell responses in *plt* mice are markedly prolonged. T cells from LNs and spleens of *plt* mice proliferate and express IL-2 in response to antigens for at least 8 wk after immunization, whereas this response in WT mice has largely subsided after 20 d (Figs. 4 and 5). We have also observed a prolonged increase in number of $V\beta8.2^+$ T cells in the draining LNs of *plt* mice in response to viral superantigens when infected with mouse mammary tumor virus (32; and H. Nakano, unpublished observation).

Associated with these abnormalities in immune response, *plt* mice display several abnormalities in leukocyte migration after antigen challenge. First, *plt* mice demonstrate no increase in the number of T and B cells within draining LNs in the first 4 d of an immune response (Fig. 1 B). After day 4 after immunization, T and B cell numbers in draining *plt* LNs increase markedly (Fig. 1 B). This implies that a second phase of T cell expansion occurs in immunized LNs that is dependent on neither CCL21 nor CCL19. The increase in T cell numbers during this phase may be due to the local proliferation of T cells, to T cell emigration from the blood, and to the entry of T cells into immunized LNs via lymphatics. The poor migration of T cells into immunized LNs from the blood and the effective migration of these cells into LNs from the foot pad suggest that lymphatics are the predominant route by which T cells enter *plt* LNs after immunization (Fig. 7).

The second leukocyte migration abnormality seen in immunized *plt* mice is the abnormal trafficking of DCs. *plt* mice demonstrate a decreased accumulation of DCs in draining LNs after immunization (Fig. 1 B) that is similar to the defect seen after contact sensitization with FITC (25). Although DCs have been demonstrated to enter the lymphatics of *plt* mice, the assay used is not quantitative and the proportion of DCs that enter the lymphatics of *plt* mice after immunization is not known. Decreased DC entry into the lymphatics of *plt* mice is suggested by the decreased expression of CCL21 at this site (12). *plt* mice also appear to have a defect in the migration of DCs from the subcapsular space into the LN cortex (25). Thus, it is possible that a portion of the DCs mobilized from the peripheral tissues of *plt* mice bypass draining LNs, enter the circulation, and are deposited in the spleen. This is one possible explanation for the increased accumulation of these cells in spleen (Fig. 1 C) and the increased T cell proliferation in this organ after immunization. We have attempted to examine the direct

migration of labeled DCs from sensitized skin to spleen, but have been unable to detect such a phenomenon (data not shown). This negative result does not rule out the migration of DCs from skin to LNs in *plt* mice, but suggests that the frequency of labeled or Ag-bearing DCs among splenocytes is low, and that the increase in DC numbers seen in *plt* mice after immunization may be due to a nonspecific mobilization of these cells from peripheral sites.

plt mice also demonstrate an abnormality in leukocyte localization in that T cell–DC interactions occur outside the normal confines of the T cell zone (Fig. 2). In *plt* LNs, T cells and DCs colocalize in the very superficial cortex adjacent to the subcapsular sinus. In *plt* spleen, T cells and DCs colocalize in bridging channels that span openings in the marginal zone between red and white pulp. Although our studies do not specifically identify T cells responding to Ag, the concentration of T cell–DC interactions at the above locations suggest that these are the sites where Ag presentation occurs. These data also suggest that the expression of CCL19 by DCs is not essential for T cell–DC interactions, as *plt* mice lack a functional CCL19 gene (12, 14). As we have shown previously, splenic bridging channels and the superficial cortex of LNs are the sites where most DCs are found in these tissues of nonimmunized *plt* mice (25). This would suggest that these areas represent points at which the normal migration of DCs into T cell zones is blocked in *plt* mice. The accumulation of T cells at these sites after immunization may represent a similar blockade in a population of cells following the same migration route. Alternatively, activated DCs may be capable of attracting T cells to areas in which they, the DCs, are concentrated.

At present, there are several possible explanations for the abnormal immune responses observed in *plt* mice. First, the *plt* phenotype may be due to an altered kinetics of immune response. Defects in leukocyte migration may lead to a decreased intensity of antigen presentation but an increase in the period of time over which antigen presentation occurs. This may explain the persistence of activated T cells in *plt* mice in that intensity of antigenic stimulation has been demonstrated to affect the magnitude of T cell contraction (33, 34). This may also explain the sensitivity of *plt* mice to viral infection (25), as they may not begin to clear virus until substantial viral loads have developed. Second, the abnormal location of T cell priming may alter immune responses in *plt* mice. Aside from assisting in the efficient selection and stimulation of Ag-specific T cells, it is likely that T cell zones provide cytokines or growth factors that influence the development of T cell response (33). Because *plt* mice prime T cells outside this environment, these cells may not receive stimuli that normally contribute to a properly regulated immune response. Finally, it is possible that the abnormalities seen in *plt* mice are due to an alteration in antigen-presenting cells. T cell priming in *plt* mice appears to occur in areas that normally contain $CD8^{-}CD11b^{+}$ "myeloid" DCs rather than $CD8^{+}CD11b^{-}$ "lymphoid" DCs (35). This suggests that antigen presentation in *plt* mice may occur predominately via newly migrated myeloid DCs rather than interdigitating lymphoid DCs. Whether representing a separate lineage or merely a different activation state, lymphoid DCs have been regarded as possible regulatory cells. These cells have been shown to induce apoptosis of antigen-specific T cells after transient proliferation (36, 37). Thus, antigen presentation by activated myeloid DCs is consistent with the enhanced T cell responses in *plt* mice. The finding that survival of activated superantigen-specific T cells is prolonged in *plt* mice supports this possibility, as superantigens normally induce prompt apoptosis of antigen-specific T cells (38). We did not specifically distinguish these two DC populations in our localization studies, as DEC-205 can be expressed by both cell types under activated condition (39–41). Further characterization of the DC populations in *plt* mice and the response of T cells stimulated by these DCs are underway.

It is possible that the prolonged activation of T cells in *plt* mice leads to other alterations in immune response. As an example, we have found that levels of OVA-specific IgG_{2a} in *plt* mice are enhanced for up to 5 mo after immunization. However, levels of OVA-specific IgG_1, IgM, and IgG_{2b} are altered in a more complex manner, suggesting a possible alteration in the balance of Th1 versus Th2 cytokines. A characterization of humoral responses in *plt* mice will be reported separately (unpublished observations).

Our findings of enhanced T cell responses in *plt* mice are unexpected given the reported lack of primary T cell responses in $CCR7^{-/-}$ mice. Although T cell responses in $CCR7^{-/-}$ mice have not been characterized as thoroughly as those in *plt* mice, it appears that those in *plt* mice are more robust. The reason for this is most likely related to differences in T cell and DC migration. In general, abnormalities in leukocyte migration are more severe in $CCR7^{-/-}$ than in *plt* mice (22, 25, 27). The migration of activated DCs is decreased to a lessor extent in *plt* than in $CCR7^{-/-}$ mice, probably due to the preserved expression of CCL21-leu in their lymphatic endothelium (12). Low level expression of CCL21 may stimulate the entry of DCs into *plt* LNs at a level that is sufficient to support primary T cell responses. In mice lacking CCR7, this function is absent, and T cell responses are likely to be more severely impaired. Alternatively, it remains possible that a gene other than CCL21 and CCL19 is responsible for the differences observed in *plt* and $CCR7^{-/-}$ mice. Although we have partially characterized the *plt* deletion and confirmed the presence of most genes known to be in this region (Gunn, M.D., unpublished observations), we cannot rule out the possibility that an unknown gene is present in this region, is deleted in the *plt* mutation, and leads to some of the abnormalities we observe.

In summary, we demonstrate that *plt* mice, which lack expression of CCL21 and CCL19 in lymphoid organs, are capable of mounting T cell immune responses in spite of severe defects in T cell and DC migration and localization. In addition, T cell activity in *plt* mice persists at high levels for at least 8 wk after immunization. The mechanisms underlying these abnormalities are not yet known, but may involve defects in the localization of specific DC popula-

tions. *plt* mice should prove to be an excellent model to study the manner in which chemokines may regulate the magnitude, timing, and quality of immune response.

We thank Drs. A. Matsuzawa, F. Ishikawa, and Y. Okada, and Ms. Y. Tanaka for their help and advice, and M. Okuda and T. Kanai for their encouragement.

This study was supported by the Welfide Medical Research Foundation and by the Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan to H. Nakano (09770332) and T. Kakiuchi (10670606).

Submitted: 9 June 2000
Revised: 5 December 2000
Accepted: 6 December 2000

References

1. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature*. 392:245–252.
2. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science*. 290:92–97.
3. Cyster, J.G. 1999. Chemokines and cell migration in secondary lymphoid organs. *Science*. 286:2098–2102.
4. Cyster, J.G. 2000. Leukocyte migration: scent of the T zone. *Curr. Biol*. 10:R30–R33.
5. Melchers, F., A.G. Rolink, and C. Schaniel. 1999. The role of chemokines in regulating cell migration during humoral immune responses. *Cell*. 99:351–354.
6. Loetscher, P., B. Moser, and M. Baggiolini. 2000. Chemokines and their receptors in lymphocyte traffic and HIV infection. *Adv. Immunol*. 74:127–180.
7. Ansel, K.M., L.J. McHeyzer-Williams, V.N. Ngo, M.G. McHeyzer-Williams, and J.G. Cyster. 1999. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J. Exp. Med*. 190:1123–1134.
8. Yoshida, R., M. Nagira, M. Kitaura, N. Imagawa, T. Imai, and O. Yoshie. 1998. Secondary lymphoid-tissue chemokine is a functional ligand for the CC chemokine receptor CCR7. *J. Biol. Chem*. 273:7118–7122.
9. Campbell, J.J., E.P. Bowman, K. Murphy, K.R. Youngman, M.A. Siani, D.A. Thompson, L. Wu, A. Zlotnik, and E.C. Butcher. 1998. 6-C-kine (SLC), a lymphocyte adhesion-triggering chemokine expressed by high endothelium, is an agonist for the MIP-3beta receptor CCR7. *J. Cell Biol*. 141: 1053–1059.
10. Gunn, M.D., K. Tangemann, C. Tam, J.G. Cyster, S.D. Rosen, and L.T. Williams. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. USA*. 95:258–263.
11. Vassileva, G., H. Soto, A. Zlotnik, H. Nakano, T. Kakiuchi, J.A. Hedrick, and S.A. Lira. 1999. The reduced expression of 6Ckine in the *plt* mouse results from the deletion of one of two 6Ckine genes. *J. Exp. Med*. 190:1183–1188.
12. Nakano, H., and M. Gunn. 2001. Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of SLC and ELC genes in the *plt* mutation. *J. Immunol*. In press.
13. Ngo, V.N., H.L. Tang, and J.G. Cyster. 1998. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J. Exp. Med*. 188:181–191.
14. Luther, S.A., H.L. Tang, P.L. Hyman, A.G. Farr, and J.G. Cyster. 2000. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the *plt/plt* mouse. *Proc. Natl. Acad. Sci. USA*. 97:12694–12699.
15. Sallusto, F., P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C.R. Mackay, S. Qin, and A. Lanzavecchia. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol*. 28:2760–2769.
16. Dieu, M.C., B. Vanbervliet, A. Vicari, J.M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med*. 188:373–386.
17. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 401: 708–712.
18. Sallusto, F., B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol*. 29:1617–1625.
19. Pachynski, R.K., S.W. Wu, M.D. Gunn, and D.J. Erle. 1998. Secondary lymphoid-tissue chemokine (SLC) stimulates integrin alpha 4 beta 7-mediated adhesion of lymphocytes to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) under flow. *J. Immunol*. 161:952–956.
20. Campbell, J.J., J. Hedrick, A. Zlotnik, M.A. Siani, D.A. Thompson, and E.C. Butcher. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science*. 279:381–384.
21. Nakano, H., T. Tamura, T. Yoshimoto, H. Yagita, M. Miyasaka, E.C. Butcher, H. Nariuchi, T. Kakiuchi, and A. Matsuzawa. 1997. Genetic defect in T lymphocyte-specific homing into peripheral lymph nodes. *Eur. J. Immunol*. 27:215–221.
22. Nakano, H., S. Mori, H. Yonekawa, H. Nariuchi, A. Matsuzawa, and T. Kakiuchi. 1998. A novel mutant gene involved in T-lymphocyte-specific homing into peripheral lymphoid organs on mouse chromosome 4. *Blood*. 91:2886–2895.
23. Stein, J.V., A. Rot, Y. Luo, M. Narasimhaswamy, H. Nakano, M.D. Gunn, A. Matsuzawa, E.J. Quackenbush, M.E. Dorf, and U.H. von Andrian. 2000. The CC chemokine thymus-derived chemotactic agent 4 (TCA-4, secondary lymphoid tissue chemokine, 6Ckine, exodus-2) triggers lymphocyte function–associated antigen 1–mediated arrest of rolling T lymphocytes in peripheral lymph node high endothelial venules. *J. Exp. Med*. 191:61–76.
24. Warnock, R.A., J.J. Campbell, M.E. Dorf, A. Matsuzawa, L.M. McEvoy, and E.C. Butcher. 2000. The role of chemokines in the microenvironmental control of T versus B cell arrest in Peyer's patch high endothelial venules. *J. Exp. Med*. 191:77–88.
25. Gunn, M.D., S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuzawa, L.T. Williams, and H. Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med*. 189:451–460.
26. Ngo, V.N., H. Korner, M.D. Gunn, K.N. Schmidt, D.S. Riminton, M.D. Cooper, J.L. Browning, J.D. Sedgwick, and

J.G. Cyster. 1999. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J. Exp. Med.* 189:403–412.
27. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell.* 99:23–33.
28. Metlay, J.P., M.D. Witmer-Pack, R. Agger, M.T. Crowley, D. Lawless, and R.M. Steinman. 1990. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.* 171: 1753–1771.
29. Steinman, R.M., M. Pack, and K. Inaba. 1997. Dendritic cells in the T-cell areas of lymphoid organs. *Immunol. Rev.* 156:25–37.
30. Arbones, M.L., D.C. Ord, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D.J. Capon, and T.F. Tedder. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity.* 1:247–260.
31. Steeber, D.A., N.E. Green, S. Sato, and T.F. Tedder. 1996. Lymphocyte migration in L-selectin-deficient mice. Altered subset migration and aging of the immune system. *J. Immunol.* 157:1096–1106.
32. Yoshimoto, T., H. Nagase, H. Nakano, A. Matsuzawa, and H. Nariuchi. 1996. Deletion of $CD4^+$ T cells by mouse mammary tumor virus (FM) superantigen with broad specificity of T cell receptor beta-chain variable region. *Virology.* 223:387–391.
33. Sprent, J., D.F. Tough, and S. Sun. 1997. Factors controlling the turnover of T memory cells. *Immunol. Rev.* 156:79–85.
34. Hosken, N.A., K. Shibuya, A.W. Heath, K.M. Murphy, and A. O'Garra. 1995. The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J. Exp. Med.* 182:1579–1584.
35. Pulendran, B., J. Lingappa, M.K. Kennedy, J. Smith, M. Teepe, A. Rudensky, C.R. Maliszewski, and E. Maraskovsky. 1997. Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of dendritic cell subsets in FLT3 ligand-treated mice. *J. Immunol.* 159:2222–2231.
36. Suss, G., and K. Shortman. 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand– induced apoptosis. *J. Exp. Med.* 183:1789–1796.
37. de St Groth, B.F. 1998. The evolution of self-tolerance: a new cell arises to meet the challenge of self-reactivity. *Immunol. Today.* 19:448–454.
38. Webb, S.R., and J. Sprent. 1993. Factors controlling the reactivity of immature and mature T cells to Mls antigens in vivo. *Immunol. Rev.* 131:169–188.
39. Vremec, D., and K. Shortman. 1997. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J. Immunol.* 159:565–573.
40. Anjuere, F., P. Martin, I. Ferrero, M.L. Fraga, G.M. del Hoyo, N. Wright, and C. Ardavin. 1999. Definition of dendritic cell subpopulations present in the spleen, Peyer's patches, lymph nodes, and skin of the mouse. *Blood.* 93:590–598.
41. Iwasaki, A., and B.L. Kelsall. 2000. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J. Exp. Med.* 191:1381–1394.