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Neuritogenic Effects of T Cell-Derived IL-3 on Mouse Splenic Sympathetic Neurons In Vivo

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To determine the role played by lymphocytes and cytokines in the growth of sympathetic neurons in vivo, the innervation and cytokine levels were examined in the spleens of SCID mice that lack T and B cells. Splenic noradrenaline, nerve growth factor (NGF), and IL-1β levels were elevated in SCID mice. Immunohistochemical examination revealed that the density of tyrosine hydroxylase-positive (TH+) fibers of splenic central arteries in SCID mice was increased compared with wild-type C.B-17 mice, while SCID mice had significantly fewer TH+ fibers in their periarteriolar lymphatic sheaths (PALS). Two weeks after SCID mice were injected with C.B-17 splenic T cells, their TH+ fiber staining increased in the PALS. IL-3 levels increased significantly in SCID mice following T cell reconstitution, and the administration of anti-IL-3 Ab blocked the above T cell-induced increase in innervation in the PALS. Anti-IL-3 treatment also inhibited the regeneration of splenic sympathetic neurons in C.B-17 mice after they were chemically sympathetomized with 6-hydroxypipamine. Depletion of NK cells by anti-asialo GM1 promoted the splenic innervation in SCID mice, while there were no significant changes in the innervation between CD8+ T cell-deficient β2-microglobulin knockout mice and their wild type. Our results suggest that T cells (probably CD4+ Th cells but not CD8+ CTLs) play a role in regulating the sympathetic innervation of the spleen; this effect appeared to be mediated, at least in part, by IL-3. On the contrary, NK cells may exert an inhibitory effect on the sympathetic innervation.

Evidence suggests the existence of an important functional link between the nervous and immune systems (1, 2). Primary and secondary lymphoid organs are well-innervated by sympathetic efferents, and their neurotransmitter noradrenaline was reported to modulate a variety of immune functions by binding to adrenergic receptors on immune cells (3–6). Conversely, the immune system appears to influence the activity of sympathetic neurons locally and centrally by releasing cytokines that bind to receptors on neuronal and/or non-neuronal cells (7–10).

We previously examined whether lymphoid tissues could induce neurite outgrowth from sympathetic ganglia by coculturing rodent superior cervical ganglia with lymphoid tissue explants (11, 12). Preferential neurite outgrowth was observed toward thymic, splenic, and to a lesser extent, mesenteric lymph node tissue; this outgrowth seemed to be dependent on the presence of nerve growth factor (NGF). Our data also suggested that IL-1β was involved in NGF-mediated neuritogenesis. Interestingly, when mesenteric lymph node cells, isolated from gastrointestinal nematode (Nippostrongylus brasiliensis)-infected rodents were cocultured with ganglia, we found that they enhanced neurite outgrowth independent of NGF, but dependent in part in the presence of IL-3, IL-6, and GM-CSF. These in vitro data suggest that rodent lymphoid tissues can induce sympathetic neurite outgrowth, and that this effect may be mediated by various cytokines. However, the role played by cytokines on neuronal differentiation, survival, and function in vivo is still unclear.

The spleen is well-suited for the histological examination of the interaction between nerve fibers and immune cells. The splenic nerves, which predominantly (~97%) consist of postganglionic sympathetic fibers, arise from the superior mesenteric/celiac ganglion, and enter the spleen at the hilar region along with the vasculature. Some of these fibers arborize with the capsular/trabecular system. Extensive arrays of fibers also travel with the central artery and its arteriolar branches in the white pulp, and extend into the plexa that are rich in T cells (13, 14). Electron microscopic studies revealed membrane/membrane apposition between nerve terminals and T cells in the spleen (15). Macrophages and B cells that reside in the marginal zone and outer marginal sinus also appear to be sympathetically innervated, while follicular B cells appear to be sparsely innervated. The red pulp contains scattered fibers (~1% of the splenic innervation) that are primarily associated with the plexuses in trabeculae, and the sinuses. These histological findings suggested that immune cells in general, and T cells specifically, might play an active role in directing splenic sympathetic innervation. This hypothesis was further supported by data that showed that the growth of sympathetic fibers into the spleen occurred concurrently with the intrasplenic migration of T cells (16).
CD8 and T cells as a result of a spontaneous autosomal recessive mutation that is located on chromosome 16 that blocks the expression of functional cell surface Ag receptors (17, 18). However, effector cells of the innate immune system including granulocytes, macrophages, and NK cells are normal in these animals, and NK cells form the major cellular constituent in the SCID spleen (19-22).

SCID mice lack functional T and B cells as a result of a spontaneous autosomal recessive mutation that is located on chromosome 16 that blocks the expression of functional cell surface Ag receptors (17, 18). However, effector cells of the innate immune system including granulocytes, macrophages, and NK cells are normal in these animals, and NK cells form the major cellular constituent in the SCID spleen (19-22). SCID mice have been extensively used to study the host response to viral, bacterial, and parasitic pathogens. They have also been used to examine the effects of immune regulation on the nervous system. Serpe et al. (23), using a peripheral nerve lesion model, showed that facial motoneuron loss was exacerbated in SCID mice, an effect that was reversed by T and B cell reconstitution. Mitchell et al. (24) examined the innervation of the thymus using tyrosine hydroxylase (TH)-directed immunohistochemistry (TH is the rate-limiting enzyme in noradrenaline synthesis, and a specific marker of catecholaminergic neurons). TH+ immunoreactive nerve fibers (TH+ fibers) were detected in the perivascular and subcapsular regions and, to a lesser extent, in underlying cortical regions of the thymus of BALB/c mice (the original strain of SCID mice). A similar but denser pattern of innervation was found in SCID mice. Thymic repopulation of SCID mice following bone marrow transplantation restored their innervation density to normal, suggesting that lymphocytes play a role in thymic innervation.

The purpose of this study was to examine the effects of lymphocytes and their cytokine effector molecules on splenic sympathetic neurite outgrowth in vivo in SCID mice. We examined the innervation and cytokine levels in the spleens of SCID mice, with or without reconstitution with C.B-17 splenic T or B cells and depletion of NK cells by an anti-asialo GM1 Ab (22). We also examined the splenic innervation of β2-microglobulin knockout (B₂m KO) mice which are deficient in MHC class I-restricted CD8+ T cells (25). Furthermore, we determined the effects of various cytokines on the regeneration of splenic nerves following chemical sympathectomy in normal mice. Our results suggest that T cells (maybe CD4+ T cells but not CD8+ CTLs) regulate the sympathetic innervation of the spleen, and that IL-3 plays a central role in mediating this effect.

Materials and Methods

Mice

Mice were bred and housed in filter-capped isolator cages in our specific pathogen-free animal care facility (Osaka Prefecture University, Osaka, Japan). Ten- to 20-week-old male and female C.B-17 +/- (C.B-17) and C.B-17 scid/scid (SCID) mice (Clea Japan) were used for these experiments. Male and female B₂m KO were provided by Prof. T. Inaba (Osaka Prefecture University, Osaka, Japan) and were used at 8-13 wk of age. C57BL/6 mice (Clea Japan) were used at 8-21 wk of age. Sampling and injection were performed between 10 and 12 a.m. The study was approved by the Osaka Prefecture University Animal Care and Use Committee.

Immunohistochemical staining for TH and NGF

Mice were deeply anesthetized with ether, after which they were perfusion fixed with either 4% buffered paraformaldehyde (for TH staining) or zamboni solution (for NGF staining) by cardiac puncture. Spleens were removed, postfixed, and washed with phosphate buffer. The tissues were cytoprotected in 5% sucrose for 1 h followed by 10% sucrose overnight. Serial 10-μm sections were then cut using a freezing microtome, after which they were washed in phosphate buffer containing 0.1% Triton X-100. The sections were rinsed three times with phosphate buffer, and then had their endogenous peroxidase activity inhibited by treatment with 3% H₂O₂ at room temperature (RT) for 10 min. After they were rinsed, the sections were blocked with 1.5% skim milk at RT for 15 min, after which they were incubated with either an affinity-purified rabbit anti-rat TH Ab (1/400 dilution; Chemicon International) or monoclonal rabbit anti-mouse NGF Ab (400 pg/ml; Santa Cruz Biotechnology) for 2 days at 4°C. After rinsing, the sections were incubated with a FITC-labeled donkey anti-rabbit IgG Ab (1/400 dilution; Jackson ImmunoResearch Laboratories) at RT for 1 h. After rinsing, the sections were mounted with glycerine and observed under confocal microscope system (C1Plus; Nikon). Serial 0.35-μm areas were scanned as a stack of a single image slice along the vertical (Z) axis of the section.

ELISA for noradrenaline, NGF, and other cytokines

Spleens (20–40 mg) were placed in 1 ml of 100 mM Tris-HCl containing 2% BSA, 1 M NaCl, 4 mM EDTA/2 Na, 2% Triton X-100, 0.1% NaCN, 17 μg/ml PMSF, and 20 μl of protease inhibitor mixture (Sigma-Aldrich), after which they were homogenized on ice and centrifuged at 14,000 rpm at 4°C for 30 min. The supernatants were then collected and assayed by ELISA for the presence of murine 2.5S NGF (Chemicon International), murine IL-1β (BioSource International), murine IL-2 (BioSource International), murine IL-6 (BioSource International), and murine GM-CSF (BioSource International). Results were expressed per milligram of splenic tissue.

Adoptive transfer of T and B cells

Spleens were aseptically removed from C.B-17 mice and dissociated in Eagle’s MEM. Spleen cells were incubated in MEM containing 10% FCS at 37°C for 1 h in a 100-mm tissue-culture plastic dish (Iwaki Glass), after which the nonadherent cells were collected and treated with distilled water to lyse the RBCs. For T cell purification, the cell suspension was then loaded onto T cell-enrichment columns (G-T), which were packed with glass beads coated with monocyte-directed IgGs (for monocyte depletion) or anti-Ig Abs (for B cell depletion). The column elute contained highly enriched T cells (>90% purity), which was confirmed by immunocytochemistry using affinity-purified rat anti-mouse CD3 (1:1000 dilution; Celltech Laboratories) and biotinylated goat anti-rat IgG (1:500 dilution; Celltech Laboratories). B cells were purified by negative selection using a mouse B cell enrichment mixture (StemCell Technologies) that contained Abs directed against mouse CD4, CD8, CD11b, myeloid differentiation Ag Gr-1, and erythroid Ag TER 119, as well as dense particles that were covalently coated with a second Ab. B cell purity was found to be >90%, which was confirmed by immunocytochemistry using mouse IgG (F(ab’)2, 1/100 dilution; Amersham Biosciences). Cell viability in both cases was found to be >85% as assessed by the trypan blue exclusion test. The cells were suspended at a concentration of 1 x 10⁷ cells/ml in serum-free MEM, and 0.5 ml were injected i.p. into SCID mice.

Two weeks later, immunohistochemistry was performed on the spleens of these animals using anti-CD3, anti-IgG F(ab’)2, and TH Abs. FACS was also performed using splenic cells prepared as mentioned above. To eliminate possible binding to FcRs, 50 μl of cell suspension was preincubated with 1 μl of rat anti-mouse CD16/CD32 mAb (0.5 mg/ml; BD Pharmingen) for 5 min on ice. Cells were incubated with 1.25 μl of CyChrome-conjugated monoclonal rat anti-mouse CD3 (0.2 mg/ml; BD Pharmingen) or CyChrome-conjugated mouse isotype-matched control mAb (0.2 mg/ml; BD Pharmingen) for 20 min on ice. After washing, the cell suspension was analyzed on a FACSort (BD Biosciences) using CellQuest software.

For the IL-3 neutralization, affinity-purified goat anti-mouse IL-3 (100 μg/mouse; R&D Systems) was injected i.p. into SCID mice 1, 5, 9 and 13 days after T cell transfer.

Chemical sympathectomy

6-Hydroxydopamine (6-OHDA; Sigma-Aldrich) was dissolved in saline containing 0.4% ascorbic acid, and 1 ml was injected into C.B-17 mice (250 mg/kg body weight). TH-directed immunohistochemistry was then performed on the spleens of these animals that were harvested 1, 3, 5, 10, and 21 days after injection. To investigate whether the effect of 6-OHDA

After rinsing, the sections were incubated with biotinylated goat anti-rabbit IgG (1/300; Vector Laboratories) at RT for 1 h. After the sections were washed, the ABC method was used to visualize the staining, using an avidin-biotin peroxidase complex kit Vectastain (Vector Laboratories) with diaminobenzidine (Wako Pure Chemical). The sections were washed with H₂O₂ to stop the reaction, after which they were processed for mounting. The sections were photographed using a digital camera (DS camera head and camera control unit; Nikon), and the degree of positive TH+ staining was calculated using NIH image analysis software (version 1.63; Research Services Branch, National Institute of Mental Health, Bethesda, MD).

For the confocal observation, serial 25-μm sections of the spleens were cut, after which they were incubated with 1% 3,3% H₂O₂ at room temperature (RT) for 10 min. After they were rinsed, the sections were incubated with biotinylated goat anti-rabbit IgG Ab (1/400 dilution; Jackson ImmunoResearch Laboratories) at RT for 1 h. After rinsing, the sections were mounted with glycerine and observed under confocal microscope system (C1Plus; Nikon). Serial 0.35-μm areas were scanned as a stack of a single image slice along the vertical (Z) axis of the section.
is drug specific, mice were injected with desipramine, which blocks the uptake of 6-OHDA into nerve fibers, before 6-OHDA injection (26, 27). Desipramine (Sigma-Aldrich) was dissolved in saline, and was injected i.p. into C.B-17 mice 30 min before 6-OHDA injection (30 mg/kg body weight).

For the cytokine neutralization study, affinity-purified goat anti-mouse IL-3 (100 μg/mouse; R&D Systems) or affinity-purified goat anti-mouse IL-6 (100 μg/mouse; R&D Systems) was injected i.p. into SCID mice 1 and 5 days after 6-OHDA treatment. Control animals received affinity-purified goat IgG (100 μg/mouse; R&D Systems). On day 10, TH-directed immunohistochemistry was performed on the spleens of these animals.

**In vivo NK cell depletion**

Normal rabbit IgG (prepared by 50% ammonium sulfate precipitation followed by dialysis with PBS (pH 7.2)) or rabbit IgG containing a mouse/rat anti-asialo GM1 Ab (Cedarlane Laboratories) was dissolved in saline, and was injected i.p. into SCID mice (20 μl/mouse). Two days later, the efficacy of such treatment was evaluated by the standard YAC-1 cell cytotoxicity assay (28), and TH-directed immunohistochemistry was performed on the spleens of these animals.

**Statistical analysis**

All data are presented as the means ± SE. Data were analyzed using an ANOVA followed by Scheftee’s F tests for multiple comparisons. A Student t test was used to determine significance when only two groups were compared.

**Results**

**Profile of splenic sympathetic innervation in SCID mice**

As shown in Table I, splenic noradrenaline levels were significantly higher in SCID, compared with normal C.B-17, mice. TH+ fibers formed dense plexuses in control mice that were associated with the central arteriole, that extended somewhat into the PALS (Fig. 1a). A dense array of TH+ fibers was found circumscribed around splenic arteries in SCID mice, though only a few fibers were detected in the PALS. Denser TH+ vascular staining but fewer PALS staining in SCID mice was further confirmed by confocal microscopy (Fig. 1b). The magnitude of TH+ staining in the vascular and parenchymal area was calculated individually, using an NIH image analysis program, by dividing each by either the total PALS or vascular area (Fig. 1c). Our data showed that TH+ vascular staining covered twice the area in SCID, compared with C.B-17, mice (434. 4 ± 16.21 μm² vs 176.2 ± 15.66 μm² per 10³ μm²). In contrast, TH+ PALS staining in SCID mice was about half of that seen in control mice (37.7 ± 3.74 μm² vs 70.2 ± 5.99 μm² per 10³ μm²). Thus, although total splenic noradrenaline levels were higher in SCID mice, probably due to their higher density of vascular noradrenergic fibers, their PALS innervation was actually reduced.

**Cytokine profile of the spleens of SCID mice**

Although NGF staining was nominal in the white pulp of C.B-17 mice, it was considerably enhanced in SCID mice (Fig. 2, a and b). Total splenic NGF levels were also found to be significantly elevated in SCID, compared with C.B-17, mice (422.8 ± 55.40 vs 231.4 ± 19.49 pg per mg of wet weight). Of the other cytokines examined, only IL-1β levels were significantly elevated in SCID mice (103.5 ± 13. 49 vs 36.3 ± 2.24 pg per mg of wet tissue; Fig. 3).

**Changes in splenic sympathetic innervation and cytokine levels in SCID mice by T cell reconstitution**

T and B cell reconstitution in treated SCID mice was confirmed immunohistochemically using anti-CD3 and -IgG Abs, respectively (Fig. 4a); staining in unreconstituted mice (injected only medium) was negligible. FACS analysis showed that splenocytes from T cell-reconstituted SCID mice contained a significantly elevated and partially recovered population of CD3+ cells (16.7 ± 4.02%, 2.8 ± 0.89%, 47.7 ± 2.86% of splenocytes from naive and T cell-reconstituted SCID and C.B-17 mice, respectively).

T, but not B, cell reconstitution significantly increased the magnitude of TH staining in the PALS (98.0 ± 5.54 μm² with T cells vs 65.3 ± 6.87 μm² without T cells per 10³ μm²) in SCID mice (Fig. 4b). Inversely, T cell reconstitution significantly reduced the magnitude of TH staining around arteries (331.1 ± 19.43 μm² vs 422.8 ± 55.40 μm²) in SCID mice (Fig. 4c).

**Figure 1.** a, Photomicrographs showing TH+ fibers in the white pulp of wild-type (C.B-17) and SCID mice. TH+ fibers were densely aggregated (white asterisks) around central arteries. Some TH+ fibers were found to extend into the parenchyma of the PALS in C.B-17 mice (arrows), while only very few fibers did so in SCID mice. Bar, 50 μm. b, Confocal microscopic immunofluorescent images showing the localization of TH+ fibers in the white pulp of C.B-17 and SCID mice. White asterisks show the central arteries and their branches. Bar, 50 μm. c, The magnitude of the TH+ areas in C.B-17 (□) and SCID (■) mice was estimated using NIH image analysis software. Each value represents the mean ± SE of 23 (C.B-17) and 28 (SCID) photographic images from 6 (C.B-17) and 7 (SCID) mice. *p < 0.01 compared with C.B-17.
with T cells vs 426.7 ± 22.84 µm² without T cells per 10⁴ µm²). B cell reconstitution did not change the degree of TH staining around arteries (data not shown).

Fig. 5 shows the changes in splenic cytokine levels before and after T cell reconstitution. NGF and IL-6 levels dramatically decreased following reconstitution, while IL-3 levels increased.

IL-1β and GM-CSF levels did not change significantly, though IL-1β levels did diminish somewhat.

The role of IL-3 in T cell-inducing splenic sympathetic innervation in SCID mice
Neutralizing anti-IL-3 Ab was administrated to SCID mice 1, 3, 5, 9, and 13 days after they were injected with T cells, after which their spleens were immunohistochemically stained for TH. Our data showed that such treatment significantly reduced TH fiber areas in the PALS to levels seen in naive SCID mice, compared with animals injected with control IgG (63.3 ± 3.81 µm² vs 102.3 ± 7.28 µm² per 10⁴ µm²; Figs. 6 and 4b). Similarly, TH⁺ areas in the splenic arteries were significantly reduced by IL-3 neutralization compared with control animals (286.0 ± 11.57 µm² vs 353.6 ± 16.24 µm² per 10³ µm²).

The role of IL-3 on splenic reinnervation following chemical sympathectomy
TH⁺ fibers, but not immune cells, nearly completely disappeared from the spleen 1 day after C.B-17 mice were treated with 6-OHDA (Fig. 7a). By day 5 postsympathectomy, labeling began to increase, with significant increases seen by day 10; full recovery was reached by day 21. TH⁺ fiber denervation induced by 6-OHDA was not observed when C.B-17 mice were pretreated with desipramine, a catecholamine uptake blocker that blocks 6-OHDA uptake (data not shown). Administration of a neutralizing anti-IL-3 Ab on days 1 and 5 postsympathectomy blocked the regeneration of TH⁺ fibers in the PALS as determined on day 10 (Fig. 7b). Although data were not shown, anti-IL-3 Ab also significantly blocked the regeneration of TH⁺ fibers around arteries. In contrast, treatment with an anti-IL-6 Ab had no effect on TH⁺ fiber regeneration both in the PALS and around the arteries (Fig. 7b).
Influence of NK cell or CD8⁺ T cell depletion on splenic sympathetic innervation in SCID mice

Two days after NK cell depletion in SCID mice with the administration of anti-asialo GM-1, the magnitude of TH staining in the PALS was significantly increased, compared with control IgG-treated SCID mice (80.6 ± 9.86 μm² vs 33.6 ± 3.91 μm² per 10⁴ m²; Fig. 8). TH⁺ vascular areas were also significantly enhanced by NK cell depletion compared with control IgG treatment (274.3 ± 25.28 μm² vs 153.0 ± 23.52 μm² per 10³ μm²).

Both TH⁺ vascular and TH⁺ PALS areas were not significantly different between CD8⁺ T cell-deficient B₂m KO mice and their

**FIGURE 5.** Cytokine levels in the spleen of T cell-reconstituted SCID mice. SCID mice were injected i.p. with either medium alone (●) or T cells (■), and their spleens were examined by ELISA 2 wk later. Each value represents the mean ± SE of 3–11 spleens in duplicate. *, p < 0.01 compared with medium alone.

**FIGURE 6.** Neutralizing effects of anti-IL-3 Ab injection on the T cell-induced increase in TH⁺ labeling in the PALS of SCID mice. Anti-IL-3 Ab (100 μg/day; ■) or control IgG (100 μg/day; □) was injected 1, 5, 9, and 13 days following T cell injection. On day 14, the spleens were harvested and subjected to TH immunohistochemistry. The size of the TH⁺ areas in the PALS was estimated using NIH image analysis software. Each value represents the mean ± SE of 3–11 photographic images from three mice. *, p < 0.01 compared with control IgG.

**FIGURE 7.** a, Reinnervation of the PALS of C.B-17 mice following chemical sympathectomy. Animals were injected with 6-OHDA and their spleens were then periodically assessed their degree of TH-immunoreactive staining using NIH image analysis software. *, p < 0.01 compared with day 0 (immediately before 6-OHDA injection). #, p < 0.01 compared with 1 day following 6-OHDA injection. Each value represents the mean ± SE of 33–169 photographic images from two to four mice. **FIGURE 8.** The influence of NK cell depletion in localization of TH⁺ fibers in the PALS in SCID mice. Two days after injection of anti-asialo GM1 Ab (20 μl/mouse; ■) or control rabbit IgG (20 μl/mouse; □) into SCID mice, the spleens were harvested and subjected to TH immunohistochemistry. The size of the TH⁺ areas in the PALS was estimated using NIH image analysis software. Each value represents the mean ± SE of 19–31 photographic images from two to three mice. *, p < 0.01 compared with control IgG.
wild-type B6 mice (31.2 ± 3.20 μm² vs 36.3 ± 4.92 μm² per 10⁴ μm² in PALS, and 144.6 ± 15.25 μm² vs 151.3 ± 18.63 μm² per 10⁴ μm²) around arteries.

Discussion

Our data showed that TH⁺ fibers were present around the vasculature of splenic arteries in normal mice, with some extending into the PALS; our findings support the findings of others in rodents (13–16). In contrast, SCID mice exhibited a significantly denser array of perivascular fibers, but fewer fibrous extension into the PALS. Noradrenaline determination of the spleen, another commonly used assay for the sympathetic innervation, showed significant elevation of it per spleen in SCID mice. Therefore, the total volume of sympathetic supply was higher in SCID mice, and the higher level of noradrenaline would originate mainly from the vasculature-innervating, but not from the PALS-innervating, nerve terminals.

Reports have suggested that alterations in immune function adversely affect splenic sympathetic innervation. Besedovsky et al. (29) showed that athymic nude mice had increased noradrenaline levels and fluorescent catecholamine-containing nerve fibers in their spleen compared with normal littermates. In contrast, reduced noradrenaline levels and nerve destruction were reported in the spleen of MRL-prlpr mice or AIDS mice: the former animal served as a genetic model of systemic lupus erythematosus, a disease in which the number of CD4⁺/CD8⁻ T cells is increased, and the latter is characterized by the rapid and profound lymphoproliferation and dysregulation of cytokine production accompanied by hypergammaglobulinemia and immunosuppression (30, 31). These data collectively suggested that immune cells inhibited neural growth. However, in these studies, it was unclear how the sympathetic innervation was altered, if at all, in the splenic parenchyma where nerve terminals and immune cells come into direct contact, nor was the mechanism by which the immune system affected sympathetic neurons assessed. Recently, Lorton et al. (32) reported a decline of noradrenaline levels and noradrenergic nerve density in the white pulp and the regions distal to the hilus, but increased nerve density in the red pulp and the hilar regions in the spleens of arthritic rats. This finding indicates an injury and compensatory sprouting response with disease development, and the redistribution of sympathetic nerves from the white pulp to the red pulp, and suggests a signal by cytokines and neurotrophins by activated immune cells migrated into the red pulp in arthritic rats.

Immunoreactive staining for neurotrophin NGF, which plays a crucial role in the development of the sympathetic nervous system (33), was relatively weak in the spleen of C.B-17 mice. In contrast, its staining was strong and widely distributed around the arteries and in the PALS of SCID mice, suggesting that its production is usually inhibited by the immune system in normal animals. IL-1β is known to stimulate NGF production in fibroblasts, astrocytes, and other cell types (34, 35), and we reported that it promoted sympathetic neurite outgrowth in lymphoid tissues by stimulating NGF production, using a rodent sympathetic ganglion and lymphoid tissue explant coculture system (11, 12). Because IL-1β levels were found to be higher in SCID mice, it may have been responsible, at least in part, for the elevated levels of NGF in the spleen of these animals. SCID mice are deficient in functional T and B cells due to a disruption in recombine activity that is responsible for the generation of T and B cell Ag receptors (17, 18). However, effector cells of the innate immune system including granulocytes, macrophages, and NK cells are normal in these animals; in fact, NK cells form the major cellular constituent in the SCID spleen (19–21). Thus, it is likely that the source of NGF in the SCID spleen is the NK cell. Although mast cells, monocytes/macrophages, and eosinophils were shown to store and release NGF (in addition to T and B cells), NGF production by NK cells has not yet been noticed (36–39). Splenic macrophages were shown to be the primary source of IL-1β (40, 41), and activated splenic SCID NK cells reportedly transcribe IL-1β (42). In the absence of T and B cells, macrophages and NK cells and the cytokines that they produce are thought to be critical in protecting SCID mice from microbial infection. Macrophage-derived cytokines such as TNF-α and IL-12 were reported to activate NK cells, inducing them to release IFN-γ (43). IL-1 was also shown to play a role in mediating macrophage/NK cell-dependent antimicrobial function (44, 45). Thus, IL-1 may play a role in triggering NGF release from macrophages and NK cells in the spleen of SCID mice.

One unanticipated finding of ours was that neurites did not extend into the PALS in SCID mice, even though NGF levels were high. We made two hypotheses: one is that lymphocytes secrete a NGF-independent critical factor for sympathetic innervation of the PALS. Another one is that cells (probably NK cells) in the PALS have an inhibitory effect on NGF action in SCID mice. To confirm the first one, SCID mice were reconstituted with splenic lymphocytes from C.B-17 mice, and the change in splenic innervation and cytokine levels were examined. Following reconstitution of SCID mice with T and B cells, their spleens display the expected patterns of lymphocytic distribution (46). T cell reconstitution resulted in the reduction in TH⁺ fiber staining around the arteries and an increase in the PALS in these animals. In contrast, there were no significant changes in TH⁺ fiber innervation following B cell reconstitution. These data suggest that the distribution of sympathetic fibers in the spleen of SCID mice is most directly influenced by T, rather than B, cells. These findings are supported by the demonstration that the growth of sympathetic fibers into the spleen paralleled the migration of splenic T cells (16). T cell reconstitution in our animals was associated with an increase in IL-3 levels and, on the contrary, NGF and IL-6 reduction. Treatment of animals with anti-IL-3 Ab blocked the ability of T cell reconstitution to enhance neuritogenesis, supporting its importance in this process. We previously demonstrated that IL-3 exerted a neurotrophic effect on cultured sympathetic neurons by a receptor-mediated process that involved the MAPK pathway (10). We also found that IL-3 stimulated neurons in nematode-infected lymphoid tissues in a coculture system (12). IL-3 is primarily produced by mast cells and Th cells (47, 48). Rudolphi et al. (49) reported that CD4⁺ T cells can be more easily transplanted to SCID mice than CD8⁺ T cells. In the current study, the splenic innervation of Bₘ KO mice was similar to their wild type. Bₘ KO mice cannot make functional cytotoxic CD8⁺ T cells (25). Therefore, it is likely that the Th cells rather than CTLs promote sympathetic innervation of the PALS by mediating IL-3.

SCID mice were reported to develop a progressive Pneumocystis carinii infection which eventually causes death, but adaptive transfer of CD4⁺ T cells was capable of clearing the organisms from the lung by initiating an intense inflammatory cascade associated with locally elevated cytokine levels such as IL-1α, IL-1β, IL-3, IL-6, IFN-γ, and TNF-α (50). Moreover, rapid elimination of microfilariae and high levels of IL-5 were observed in the sera of the reconstituted SCID mice (51). SCID mice restored with the CD45RBhigh/CD4⁺ T cell population, but not the CD45RBlow subset or unfractionated CD4⁺ T cells, developed colitis with severe mononuclear cell infiltrates into the colon and enhanced expression of cytokines including IL-1β, IL-6, TNF-α, and IFN-γ in colonic tissue (52, 53). Our results showed that splenic levels of NGF and IL-6 were reduced by T cell transfer for reasons remain unclear. Enhanced immunoreactivity of splenic NGF in SCID mice...
and its reduction by T cell transfer suggest that T cells might negatively regulate NGF expression in the spleen. IL-6 has been reported to exert a neurotrophic effect on catecholaminergic and other neurons with or without synergism with NGF (54–56), and to stimulate NGF production (57, 58). We also reported that IL-6, in addition to IL-3, stimulated neurons in nematode-infected lymphoid tissues, as described above (12). This evidence suggests that the decline in NGF and IL-6 in these animals contributed to the T cell-mediated reduction in splenic vascular innervation. We are currently investigating a role of IL-6 in sympathetic innervation by using human IL-6-transgenic mice (59). Further studies will be also needed to prove a role of different subsets of T cells in the interaction of cytokine profile and sympathetic innervation of the spleen.

6-OHDA is a noradrenergic-specific neurotoxin (60) that specifically destroys peripheral sympathetic nerve terminals, but not cell bodies, when administered systemically because it does not cross the blood-brain barrier. Following the significant loss of fibers in the spleen of C.B-17 mice 1 day after they were treated with 6-OHDA, reinnervation was observed 10 days later, and recovery appeared to be completed by 21 days. The 6-OHDA-induced denervation did not occur by pretreatment of catecholamine uptake blocker desipramine into mice. The reinnervation was significantly blocked by anti-IL-3; the effect was not seen with anti-IL-6 Ab treatment. Thus, IL-3 secreted by activated T cells was abolished with anti-asialo GM1 treatment and dramatic promotion of fibers in the spleen of C.B-17 mice 1 day after they were treated with 6-OHDA is promoted by direct action of IL-3 to neurons but also needed to prove a role of different subsets of T cells in the spleen.

To elucidate the second hypothesis, NK cells in SCID mice were abolished with anti-asialo GM1 treatment and dramatic promotion of their splenic sympathetic neurotogenesis was confirmed. Therefore, NK cells were suggested to play an inhibitory role in splenic sympathetic neuritogenesis was confirmed. There-in, interestingly, recent works have demonstrated that 6-OHDA could induce apoptosis of thymocytes, whose effect was suppressed by desipramine (61). Therefore, there is a possibility that regeneration of fibers is promoted by direct action of IL-3 to neurons but also may involve minor associated protection of T cells from 6-OHDA-induced apoptosis by IL-3 through an autocrine/paracrine fashion (62).

In conclusion, our results suggest that T cells play a role in the sympathetic innervation of the spleen by a mechanism that involves, at least in part, the release of IL-3. Because IL-3 is produced during various immunological disorders including pulmonary inflammation and multiple myeloma (65, 66), it may represent a link between these conditions and changes in sympathetic innervation.

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Disclosures
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