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The Effect of a Novel Immunosuppressant, FTY720, in Mice Without Secondary Lymphoid Organs

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Abstract

Purpose. FTY720 is a novel immunosuppressive agent that is thought to reduce the number of peripheral blood lymphocytes (PBL) by directing them toward secondary lymphoid organs such as the lymph nodes and Peyer's patches. We studied the effects of FTY720 on aly/aly mice that do not have either lymph nodes or Peyer's patches, as well as on splenectomized aly/aly mice.

Methods. FTY720 was orally administered by gavage (1 mg/kg) to aly/aly mice as well as to aly/+ mice with and without a splenectomy on 14 consecutive days. The number of lymphocytes was then counted using True Cell beads and flow cytometry. The number of B220-, CD3-, and CD4-positive cells was also determined. In addition, skin grafts from C3H donor mice were performed on these mice.

Results. FTY720 was effective in significantly reducing the total lymphocyte count as well as the B220-, CD3-, and CD4-positive subtypes in the peripheral blood of aly/+ mice as well as in aly/aly mice with and without a splenectomy. While we did observe allograft skin graft rejection in both the aly/+ mice as well as the aly/aly mice recipients and splenectomized aly/aly mice, the graft survival was prolonged in all groups. The skin allografts treated by FTY720 thus demonstrated fewer lymphocytic cells and less infiltration of CD4-positive cells.

Conclusions. The administration of FTY720 to mice without lymph nodes, Peyer's patches, or spleens still results in peripheral lymphopenia. In all groups, FTY720 was found to prevent the infiltration of CD4-positive cells in skin allografts while also prolonging skin allograft survival. The fate of these lymphocytes, however, is unclear.

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Key words FTY720 · aly/aly mouse · Secondary lymphoid organs

Introduction

FTY720 is a novel immunosuppressant derived by chemically repairing ISP-1 (myriocin/thermozymocidin) as an extract of the filtrate of cultured *Isaria clairii* (Ascomycotina)[1] and then synthesizing the repaired substance.[2–4] Its chemical structure is completely different from that of conventional immunosuppressants. To date, FTY720 has been reported to reduce the peripheral lymphocyte count significantly when orally administered alone. The main mechanism of action for FTY720, however, remains unclarified. To clarify this mechanism, some researchers have hypothesized that FTY720 may induce the apoptosis of lymphocytes to achieve immunosuppression.[5,6] Others have proposed that the number of lymphocytes decreases as a result of their movement toward secondary lymphoid organs such as the lymph nodes and Peyer's patches.[7] Recently, more researchers tend to support the latter hypothesis. In the present study, we evaluated the effects of FTY720 on both the peripheral lymphocyte count and skin grafting using aly/aly mice,[5] which lacked all lymph nodes and Peyer's patches, and aly/aly mice, which underwent a splenectomy and lacked all secondary lymphoid organs.

Materials and Methods

Experimental Animals

Male aly/aly mice (H2b, 6–8 weeks of age) and male aly/+ mice (H2b, 6–8 weeks of age) were used to analyze the peripheral blood. As skin-grafting models, male C3H/

HeJ mice (H2k, 6–8 weeks of age) were used as donors, while male aly/+ mice (H2b, 6–8 weeks of age), male aly/aly mice (H2b, 6–8 weeks of age), and male splenectomized aly/aly mice (H2b, 6–8 weeks of age) were used as recipients. All experimental animals were purchased from Crea Japan (Tokyo, Japan) and placed in a biologically clean room. A splenectomy was performed 1 week prior to the peripheral blood analysis and skin grafting. When performing the peripheral blood analysis, the animals were divided into the following four groups of four mice each: group I (aly/aly mice), group II (splenectomized aly/aly mice), group III (aly/+ mice), and group IV (splenectomized aly/+ mice). When performing skin grafting, they were divided into the following three groups of five mice each: group V (aly/+ mice), group VI (aly/aly mice), and group VII (splenectomized aly/aly mice).

Medication

FTY720, which was purchased from Novartis Pharma (Basel, Switzerland), was dissolved in distilled water at 4°C for preservation. Control mice were given distilled water.

Skin Grafting

The animals were given anesthesia by the intraperitoneal administration of Avertin (2,2,2-tribromethanol in tert-amyl alcohol: Sigma-Aldrich, Fisher, St. Louis, MO, USA). The animals selected as donors next underwent a bilateral resection of their ears. The resected ears were divided into two layers and the external skin was used as a skin graft to cover the recipient's back. The skin graft was fixed with sterile gauze and adhesive bandage, which circumferentially covered the trunk including the chest and back. The fixation was removed 5 days after surgery. A necrotic lesion reflecting graft rejection was observed until the affected area exceeded 90% of the skin graft.

Flow Cytometry

Blood samples were collected from the caudal vein. A Trucount tube (Becton Dickinson, San Jose, CA, USA) was used to prepare the mixture of the peripheral blood sample (30 µl) and heparin added phosphate blood saline (PBS) solution (17 µl; 0.2% bovine serum albumin, 0.1% azide, 20 U heparin). Furthermore, Fcblock (0.15 µl; mouse monoclonal antibody, PharMingen, San Diego, CA, USA) was added and the mixture was stirred for 10 min. Next, the antibody (0.15 ml) was added to each sample. The samples were shielded from the light and the mixture was stirred for 10 min. Regarding the antibodies, FITC antimouse CD3 (PharMingen) and PE antimouse CD4 (PharMingen) were used to the stain T cells, while PE antimouse CD45R/B220 (PharMingen) was used to stain the B cells. Afterwards, OptiLyse C (0.5 ml; Immunotech, Westbrook, ME, USA) was added and the mixture was stirred for 10 min. The labeled cells were analyzed by Flow Cytometry (Becton Dickinson). The following formula was used to calculate the cell count:

$$\frac{\#\text{ of events in the region containing cell population}}{\#\text{ of events in absolute bead count region}} \quad \frac{\#\text{ of beads per test}}{\text{test volume}} = \text{absolute count of cell population}$$

Histopathological Staining

After fixation in 10% formalin and paraffin embedding, the sections (4–6 µm) were prepared. Hematoxylin–eosin (H&E) staining and CD4 immunohistological staining were conducted. The sample was allowed to react with purified rat antimouse CD4 (PharMingen, diluted 1/500), as the primary antibody, at room temperature for 1 h and then with Vectastain (Vector, Burlingame, CA, USA, ABC kit), as the secondary antibody, at room temperature for 30 min. DAB (Vector, DAB kit) was used for visualization.

Statistical Analysis

The level of reduction in T cells after the administration of FTY720 was compared between the treatment groups by using analysis of variance, while graft survival was compared between them by using Student's *t*-test. The averages listed in the figures and tables were expressed by the mean ± SE. A value of less than 0.05 was considered to be statistically significant ($P < 0.05$).

Results

Effects of FTY720 on the Peripheral B220-, CD3-, and CD4-Positive Cells: Comparison Between the Pretreatment and Post-Treatment Count

A thin gastric tube was inserted into the stomach to administer FTY720 (1 mg/kg per day) consecutively from days 0 to 14. Flow cytometry was conducted to count the peripheral B220-, CD3-, and CD4-positive cells three times at days 0 (pretreatment), 7, and 14 (Fig. 1). In all the groups, the post-treatment B220-,

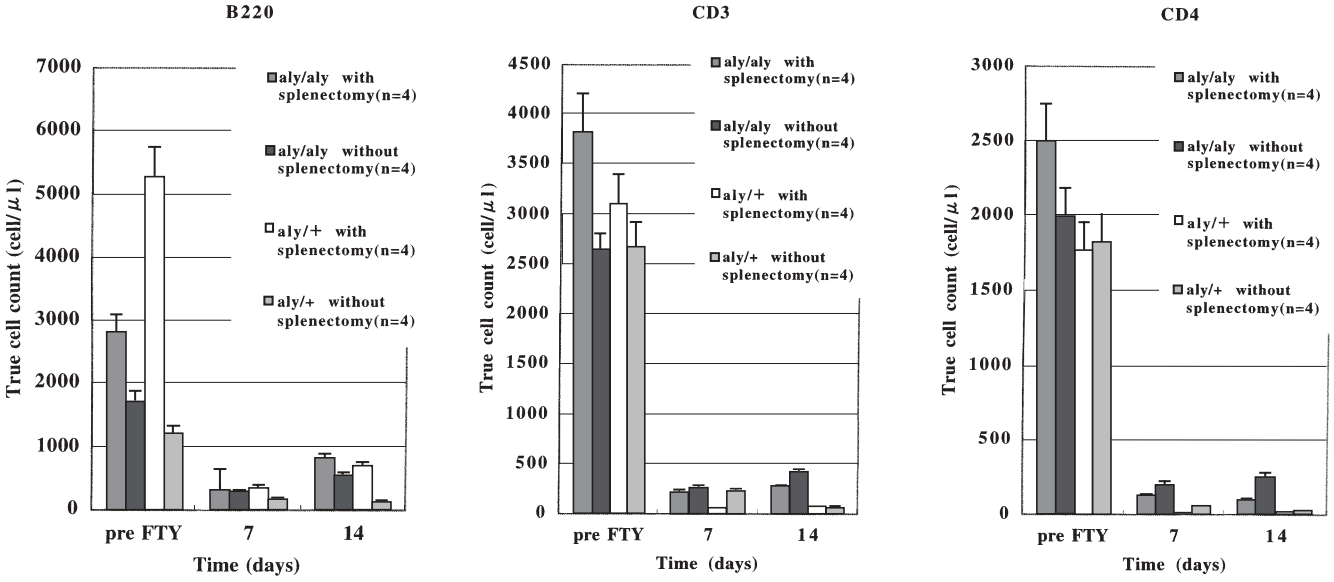

Fig. 1. Profiles of peripheral lymphocyte count after continuous administration of FTY720 (1 mg/kg per day). In all the groups, the post-treatment B220-, CD3-, and CD4-positive cell counts decreased significantly on days 7 and 14

CD3-, and CD4-positive cell count decreased significantly on days 7 and 14. T-cell count was compared between the normal aly/aly mice and aly/+ mice. According to the results obtained, in the aly/+ mice the CD4 positive cell count changed significantly on day 7, and the CD3- and CD4-positive cell count changed on day 14.

Effects of the Oral Administration of FTY720 on Graft Survival

Skin grafts collected from C3H mice were transplanted into aly/+ mice, aly/aly mice, and splenectomized aly/aly mice. FTY720 (1 mg/kg per day) was administered, by using a thin gastric tube, consecutively from the day before transplantation until the 14th day postoperatively. The administration of FTY720 contributed to a significant prolongation of the graft survival in aly/+ mice and aly/aly mice (Figs. 2 and 3A). In the splenectomized aly/aly mice, no significant difference was recognized although a prolongation of the graft survival was confirmed (Fig. 3B).

Histopathological Evaluation of Skin Grafts After the Administration of FTY720

Seven days after transplantation, the skin grafts transplanted into the aly/+ mice, aly/aly mice, and splenectomized aly/aly mice were examined to evaluate the effects of FTY720. FTY720 (1 mg/kg per day) was consecutively administered from the day before surgery until the removal of the transplanted skin graft on the

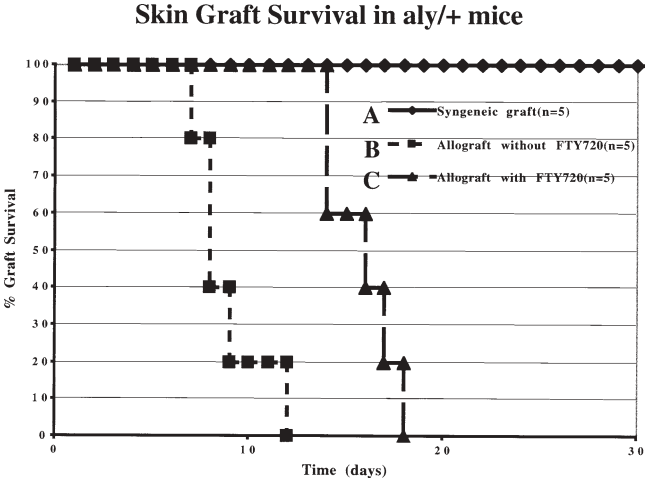

Fig. 2. Skin graft survival in the aly/+ mice. Allografts with FTY720 (*B*) versus allografts without FTY720 (*C*): $P = 0.0003$

7th day postoperatively. According to the results of the H&E staining, in the nonmedication group, mild mononuclear cell infiltration was detected in the syngeneic grafts, while epidermal thickening and a remarkable degree of mononuclear cell infiltration in the basal layer were detected in the allografts. In the FTY720 administration group, mild mononuclear cell infiltration in the basal layer was detected in the allografts. According to the results of H&E staining of the samples collected from the aly/aly mice and splenectomized aly/aly mice, as in the case of aly/+ mice, in the nonmedication group, epidermal thickening and marked mononuclear cell

Strain	FTY720	splenectomy	Graft survival(days)	Mean(days)	
aly/aly	-	-	19,21,22,23,26 (n=5)	22.2±2.6	※A
aly/aly	+	-	26,31,34,37,42 (n=5)	34.0±6.0	
aly/aly	-	+	14,14,16,18,21 (n=5)	16.6±3.0	※B
aly/aly	+	+	18,19,20,21,22 (n=5)	20.0±1.6	

Fig. 3. Skin graft survival in the aly/aly mice and splenectomized aly/aly mice. *A*, aly/aly mice with FTY720 (*row 2*) versus aly/aly mice without FTY720 (*row 1*): $P = 0.0039$; *B*, splenectomized aly/aly mice with FTY720 (*row 4*) versus splenectomized aly/aly mice without FTY720 (*row 3*): $P = 0.0536$

infiltration in the basal layer were detected in the allografts. In the FTY720 administration group, mild mononuclear cell infiltration in the basal layer was detected in the allografts. When CD4 was used for staining, in the case of aly/+ mice which received no medication, a slight expression of CD4-positive cells in the basal layer was recognized in the syngeneic grafts (Fig. 4A). A marked expression of CD4-positive cells in the basal layer was observed in the allografts (Fig. 4B). In the FTY720 administration group, a slight expression of CD4-positive cells in the basal layer was recognized in the allografts and a partial aggregation was confirmed (Fig. 4C). The findings obtained from the aly/+ mice were also recognized in aly/aly mice and splenectomized aly/aly mice which received no administration of FTY720. A marked expression of CD4-positive cells and their aggregation in the basal layer were observed. In aly/aly mice and splenectomized aly/aly mice which were administered FTY720, only a slight expression of CD4-positive cells and their partial aggregation in the basal layer were recognized in the allografts (Fig. 4D–G).

Discussion

The action mechanism of action for FTY720 remains a subject of controversy. Some researchers have hypothesized that FTY720 induced apoptosis of lymphocytes to achieve immunosuppression.[5,6] Others have proposed that the number of lymphocytes decreased as a result of the promotion of their movement toward such secondary lymphoid organs as the lymph nodes and Peyer's patches.[7] Recently, Mandala et al.[8] reported that peripheral lymphocytes were isolated not in the spleen but in the lymph nodes. Accordingly, the latter has been regarded as the dominant hypothesis. The main mechanism of action, however, remains unclear. In this study, aly/aly mice and aly/+ mice were used to compare the reduction of levels of T cells after the administration of FTY720. The CD4-positive cell count on day 7 and CD3- and CD4-positive cell count on day 14 showed significant changes. These changes seemed to be attributable to the movement of lymphocytes in aly/+ mice which had the lymph nodes as their end target. In splenectomized aly/aly mice without the secondary lymphoid organs, the administration FTY720 resulted in a reduction of peripheral lymphocytes which was also observed in the aly/+ mice and normal aly/aly mice. This finding suggests the possibility that a reduction in the number of peripheral lymphocytes following the administration of FTY720 did not result in isolation of lymphocytes in the lymph nodes and spleen. Because a splenectomy induced no changes, a new mechanism of action for FTY720 was thus identified, which is different from the former mechanism suggesting the movement of lymphocytes toward the lymph nodes and spleen.

Various graft models were used to evaluate the effects of FTY720 in animal experiments. The experimental results demonstrated that FTY720 effectively prolonged graft survival.[9,10] The infiltration of T cells into grafts is regarded as an important finding reflecting graft rejection. The expression of CD4-positive cells is particularly important[11–13] in graft rejection. Yanagawa et al.[14] reported that the inhibition of the infiltration of CD3-positive cells into grafts by FTY720 contributed to a prolongation of graft survival. In the present study, in order to conduct skin grafting we selected C3H mice as donors and aly/+ mice, aly/aly mice, and splenectomized aly/aly mice as recipients. In all the treatment groups, graft survival was prolonged after the administration of FTY720. There was a significant difference between the aly/+ mice and aly/aly mice (Fig. 4). In splenectomized aly/aly mice, no significant difference was recognized although the graft survival was prolonged. According to the results of H&E staining, in all treatment groups the administration of FTY720 inhibited mononuclear cell infiltration in the grafts. CD4 staining also demonstrated the inhibition of CD4-positive cell infiltration into the grafts in all treatment groups. The infiltration of CD4-positive cells into the skin grafts is regarded as one of the causative factors of graft rejection. FTY720 therefore appears to inhibit their infiltration into the grafts. In view of the fact that FTY720 prolonged graft survival in all the treatment groups and the changes of peripheral lymphocyte count induced by FTY720, the role of FTY720 can thus not be fully explained by

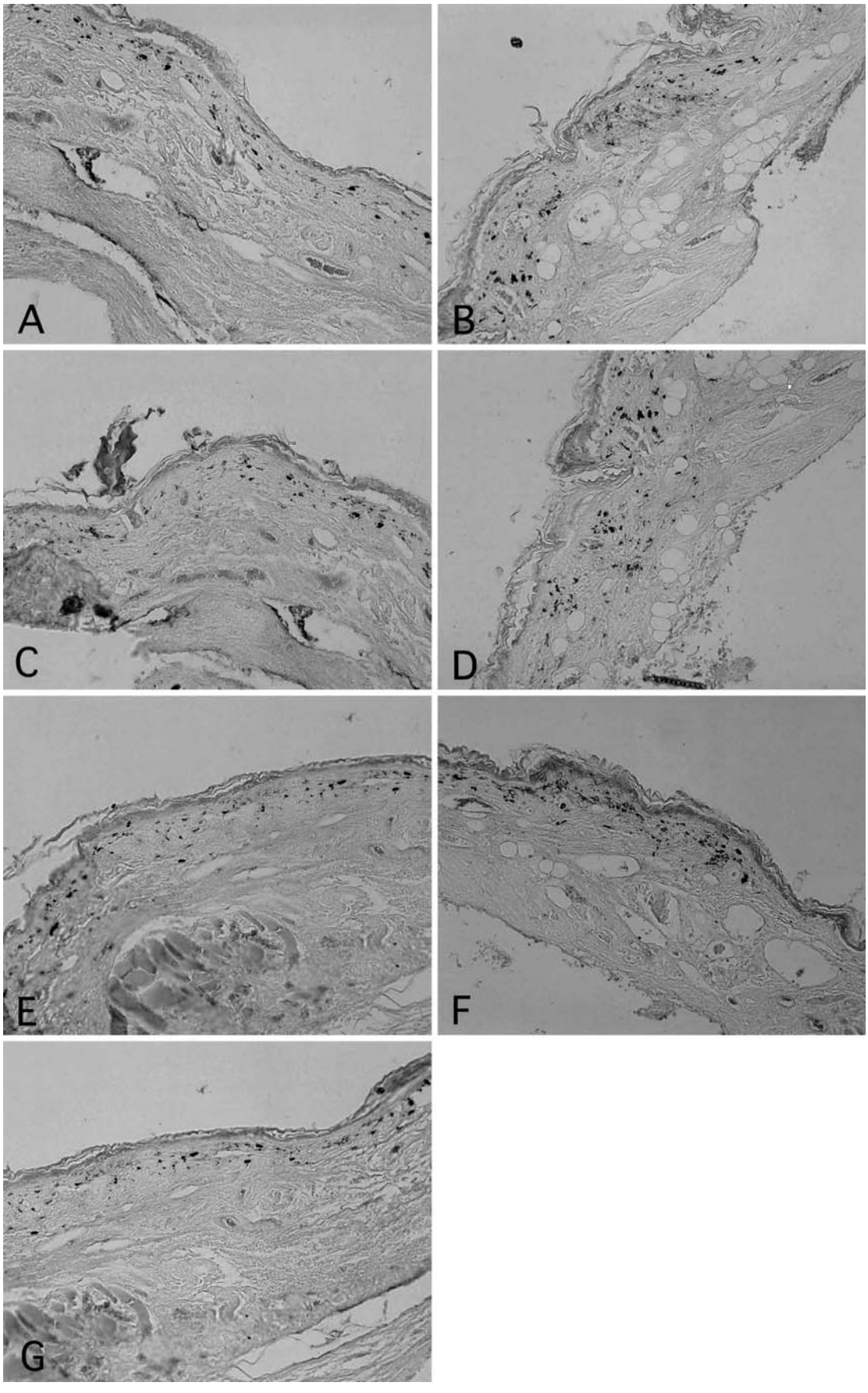
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Fig. 4. Histopathological images of the skin grafts of aly/+ mice (**A–C**; CD4 staining) and histopathological images of the skin grafts of aly/aly mice and splenectomized aly/aly mice (**D–G**; CD4 staining). **A** Syngeneic graft. **B** Allograft. **C** Allograft with FTY720. The skin graft collected from the aly/+ mouse was transplanted as a syngeneic graft into the aly/+ mouse. The skin graft collected from the C3H mouse was transplanted as an allograft into the aly/+ mouse. FTY720 was administered at a dose of 1 mg/kg once daily during the period from the day before surgery to the day of graft resection. **A** A slight expression of CD4-positive cells in the basal layer was recognized. **B** A marked expression of CD4-positive cells in the basal layer was recognized and their aggregation was also confirmed. **C** A slight expression of CD4-positive cells in the basal layer was recognized and their partial aggregation was also observed. (**A–C**: ×200). **D** aly/aly mice. **E** aly/aly mice with FTY720. **F** Splenectomized aly/aly mice. **G** Splenectomized aly/aly mice with FTY720. C3H mice were used as donors. FTY720 was administered at a dose of 1 mg/kg once daily during the period from the day before surgery to the day of graft resection. The graft was rejected on the 7th day postoperatively. In **D** and **F**, a marked expression of CD4-positive cells in the basal layer was recognized and their aggregation was confirmed. In **E** and **G**, a slight expression of CD4-positive cells in the basal layer was observed and their partial aggregation was confirmed. (**D–G**: ×200)

the hypothesis that FTY720 promotes the movement of lymphocytes and isolates them in such secondary lymphoid organs as the lymph nodes, Peyer's patches, and spleen.

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