Title

Amelioration of colitis through blocking lymphocytes entry to Peyer's patches by sphingosine-1-phosphate lyase inhibitor

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The disclosure statement

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Abstract

Background and Aim

Sphingosine-1-phosphate (S1P) receptor 1, a therapeutic target of the $S1P_1$ agonist FTY720, plays a crucial role in lymphocyte migration and is expressed in several cells including naïve T lymphocytes and endothelial cells. 2-acetyl-4-tetrahydroxybutyl imidazole (THI), an inhibitor of S1P lyase (SPL), exhibits immunomodulatory activity through increasing the S1P concentration in the secondary lymphoid organs, but its effects on colitis remain unclear. We aimed to clarify how THI affects colitis and migration of naïve T lymphocytes in Peyer's patches (PPs).

Methods

The effect of THI on gut immunity was investigated by analyzing the dextran sulfate sodium (DSS)-induced murine colitis model, lymphocyte components in thoracic duct lymphocytes (TDLs), and microscopic movement of TDLs in PPs.

Results

THI ameliorated DSS-induced colitis histologically by causing a significant decrease in colonic lymphocyte infiltration and expression of mucosal proinflammatory cytokines. THI suppressed the inflow of naïve T lymphocytes into the thoracic duct. Microscopic observation of PPs in control animals revealed that many TDLs egressed to the stroma and migrated to lymph capillaries after attaching to the high endothelial venules (HEVs). THI or FTY720 treatment in recipient animals blocked lymphocyte egression from the HEVs to the stroma.

Conclusions

This is the first study to clarify the ameliorating effects of THI on DSS-induced colitis. Microscopic observations demonstrated the involvement of HEVs in the egression of S1P-dependent gut-tropic T lymphocytes to lymph capillaries. This SPL

inhibitor might become a novel immunosuppressant for IBD therapy by blocking infiltration of lymphocytes through HEVs into the stroma in PPs.

Key words

2-acetyl-4-tetrahydroxybutyl imidazole (THI), Sphingosine-1-phosphate lyase (SPL), Peyer's patches (PPs), high endothelial venules (HEVs), lymphocyte migration

Introduction

Recirculation of naïve lymphocytes from the blood to lymphoid tissues under physiological conditions is generally regarded as a key phenomenon in the gut immunity.[1] In the presence of inflammation, antigens are presented to naïve lymphocytes by antigen-presenting cells including dendritic cells (DCs) in the stroma of secondary lymphoid organs (SLOs), such as Peyer's patches (PPs) or mesenteric lymph nodes (MLNs). The lymphocytes then flow into lymph capillaries (LCs) and enter systemic circulation. Naïve lymphocytes are circulated through the bloodstream to reach high endothelial venules (HEVs), the portal of entry into PPs. Lymphocytes interact with endothelial cells of HEVs and adhere to each other, then egress to the stroma. These interactions before egression to the stroma are regulated by adhesion molecules. After egression to the stroma, lymphocyte migration occurs through a variety of mechanisms, such as autotaxin-lysophosphatidic acid axis;[2,3] homeostatic chemokines;[4] and stromal cell transportation.[5] Sphingosine-1-phosphate (S1P), one of the migration regulators described above, plays a crucial role in lymphocyte egression from SLOs to lymphatics and is supposed to be a key modulator of lymphocyte migration in PPs.

S1P, a bioactive sphingolipid metabolite, works as an important modulator of many physiological processes, including cancer and diabetes.[6,7] S1P is secreted by a variety of cells and its function is mediated by a family of five specific G protein-coupled receptors (S1P receptor 1-5).[8,9] S1P receptor 1 ($S1P_1$) is known to be expressed predominantly on naïve T cells, activated DCs, and endothelial cells, but is negligible on gut-homing effector cells.[10] Previous studies have reported that S1P agonists such as FTY720 had ameliorating effects on intestinal inflammation.[7,11,12] Recently, $S1P_1$ agonist Ozanimod was reported to be effective in the treatment of ulcerative colitis.[12] In addition, S1P has been reported to exert

effects at other points of lymphocyte migration possibly by forming an S1P gradient in the stroma,[13] modifying endothelial tight junctions,[14] and controlling reverse transendothelial migration (RTM) via cooperation with chemokine receptors.[15] S1P has been shown to be crucial for lymphocyte migration throughout the body, but how S1P regulates lymphocyte dynamics from HEVs to LCs in the stroma of PPs has never been observed in detail.

S1P lyase (SPL) decomposes S1P into hexadecenal and phosphoethanolamine irreversibly and generates an S1P gradient that regulates lymphocyte egression from SLOs into peripheral blood. 2-acetyl-4-tetrahydroxybutyl imidazole (THI), a component of caramel food coloring, is reported to inhibit SPL, resulting in the disappearance of the S1P gradient,[13] which supposedly suppresses lymphocyte migration. It was reported that peripheral lymphocytes reduced by THI were recovered within 48 hours,[16] which was shorter than the recovery period for the suppression induced by FTY720 (approximately 4-6 weeks).[17] Therefore, THI could be administered to humans with less adverse effects. However, the mechanism is not fully understood because THI did not inhibit SPL *in vitro*,[18] and whether it ameliorates colonic inflammation remains unclear. Therefore, in this study, we aimed to clarify the effect of THI on colitis and the migration of naïve lymphocytes in PPs *in vivo*.

Methods

The experimental protocol was approved by the Animal Research Committee of National Defense Medical College (No. 16058). Animals were maintained on standard laboratory chow (CLEA Japan Inc, Tokyo, Japan). The care and use of laboratory animals were in accordance with the National Institutes of Health guidelines.

Analysis of DSS-induced colitis model

C57/BL6 male mice (8 weeks) were employed. Some mice received 3% dextran sulfate sodium (DSS, Lot No. M7191, ICN Biochemicals, Cleveland, OH, USA) dissolved in drinking water for 5 days followed by normal water for 2 days. Some mice were treated with THI (50 mg/L, Wako Pure Chemical Industries, Ltd, Osaka, Japan) in drinking water for 3 days before inducing colitis according to previous reports showing the dose enough to increase S1P concentration.[19] We examined daily symptoms of colitis by monitoring body weight, rectal bleeding, and stool consistency (defined as disease activity index, DAI). Histological damage was assessed in hematoxylin-eosin (H&E) staining by a scoring system (Cooper's score, a scale of 0 to 4).[20] We also counted the number of lymphocytes in paraffin section of the colon in proportion with the length of muscularis mucosa as described previously.[21]

mRNA expression by RT-PCR

Total RNA of whole colonic tissue was extracted as described previously.[22] Primer and probes were purchased from Applied Biosystems (Foster City. CA, USA): TNF-α (Mm00443258), IFN-γ (Mm00801778), TGF-ß (Mm03024053), IL-1ß (Mn00434226), IL-6 (Mm00446190), IL-10 (Mm00439616) and IL-17α (Mm00439619). The results were standardized to mouse GAPDH.

Investigation of lymphocyte counts in some organs

The number of the cells in some organs, such as PPs, MLNs, spleen, thymus and bone marrow (BM, collected from one leg) were investigated to see the effects of THI on altered distribution of lymphocytes using the same model as mentioned above. The organs were minced and the numbers of lymphocytes were countered by a cell counter. The ratio of $CD4^+$-cells in each group was analyzed by flow cytometry (FCM, FACS Callibur, Becton, Dickinson and company) by labeling anti-rat PE-CD4Ab (BD Biosciences, NJ, USA).

Collection and separation of lymphocytes

The thoracic duct of male Wistar rats (250-300g) was cannulated as described by Bollman et al.[23] Then animals were maintained in Bollman's cages and saline was infused into rat's duodenum from the silicon tube at a flow rate of 3 ml/h. Thoracic duct lymphocytes (TDLs) were collected in ice-cold vials containing 6 U/mL heparin, 10% fetal bovine serum, and RPMI 1640 medium (pH 7.4; GIBCO, Grand Island, NY). In some experiments, rats were treated with THI (50 mg/L) in drinking water for 3 days before cannulation.

Lymphocyte labeling with carboxyfluorescein diacetate succinimidyl ester

Carboxyfluorescein diacetate succinimidyl ester (CFDSE; Thermo Fisher Scientific, MA, USA) was dissolved in DMSO to 15.6 mM. Lymphocytes (1×10^9) in 50 ml of RPMI 1640 were incubated with 50 µl of CFDSE solution for 30 min at 37°C as described previously.[24]

Experimental setup for microvascular studies

Under continuous anesthesia with 2 % isoflurane (Wako Pure Chemical Industries), the abdomen was opened via a midline incision. About ten centimeters of the ileal segment including PPs was chosen for observation and placed on a plastic plate. The intestine was kept warm and moist with phosphate buffered saline (PBS) warmed to 37°C. Suitable areas of the microcirculation in PPs were observed

through the serosa on a confocal laser scanning microscope (CLSM, A1R+, Nikon, Tokyo, Japan). The adjacent intestinal segment and mesentery were covered with absorbent cotton soaked with PBS. Then CFSE-labeled TDLs (1×10^9 cells) were injected into the jugular vein of recipient rats over one minute. In some experiments, recipient rats were administered FTY720 (1 mg/kg, ChemScene, NJ, USA) per oral 3 hours before injecting TDLs.

Microcirculation of lymphocytes

TDLs in the microvasculature of PPs were continuously monitored on a CLSM and recorded on a computer for 3 hours in a manner of time-lapse photography at the interval of 30 seconds after injecting cells. Texas Red–dextran (25 mg/kg, Thermo Fisher Scientific, MA, USA) for staining the bloodstream and Hoechest 33342 (5 mg/kg, Thermo Fisher Scientific) for staining cell nuclei were injected into the jugular vein of the recipient rats. Lymphocytes adhering to HEVs more than 30 seconds were defined as “adhesive lymphocytes”. Lymphocytes emigrating from HEVs to stroma were defined as “migrating lymphocytes”. We calculated the average percentage of migration (migrating lymphocytes / adhesive lymphocytes + migrating lymphocytes) per field of vision (approximately 0.3 mm^2).

Characteristic evaluation of TDLs

We examined the number of T lymphocytes expressing L-selectin in TDLs by using the following antibodies: anti-rat PE-CD4 mAb and anti-rat FITC-CD62L mAb (BD Biosciences). For controls, lymphocytes were preincubated with isotype-matched, irrelevant antibodies. All incubations with antibodies were performed at 4°C for 30 min (1×10^6 lymphocytes).

Comparison of ameliorating effect between THI and FTY720

6 male C57BL/6 mice were employed in control group. Some mice received 3% DSS in drinking water for 5 days. Some mice were administered FTY720 (1

mg/kg, ChemScene, NJ, USA) intraperitoneally for 3 days before inducing colitis. Some mice were treated with THI as mentioned above. Clinical score, histological change and mRNA expression were investigated in the same way.

Effects of THI on adhesion molecules on TDLs and bEnd.3 cells

TDLs were incubated with THI (0.8, 8, and 80 μg/mL) for 3 hours. The percentage of lymphocytes expressing MRα4 integrin was examined by labelling anti-rat FITC-MRα4 integrin mAb (Bio-Rad Laboratories Inc, CA, USA) and anti-rat PE-CD4 mAb for FCM analysis.

1×10^6 mouse bEnd.3 cells (passages 6-10; ATCC) were seeded onto 6-Well Millicell Hanging Cell Culture Inserts (Millipore Corporation, Billerica, MA, USA) and cultured to confluency for 2 days at 37°C and 10% CO_2. The cell monolayers were treated with THI for an additional 3 hours. Then expression of adhesion molecules, such as MAdCAM-1 (Mm01173246), VCAM-1 (Mm00449197), and ICAM-1 (Mm00516023) were measured by quantitative RT-PCR.

Statistics

Variability of the data is expressed as the standard error of the mean (SEM). For comparative analysis in each group, homoscedasticity of them were examined using Levene's test for equality of variance. Differences between groups were examined using Student's t test in two groups with equal variance and Wilcoxon rank sum tests in multigroup with unequal variance with JMP Pro software (SAS Institute Inc, NC, USA). Values of $p<0.05$ were considered statistically significant.

212 *Results*

213 In this study, we investigated the effects of THI on DSS-induced murine
214 colitis and examined the effects on TDLs by FCM analysis. Then we observed
215 migration of naïve lymphocytes in PPs of rats on a CLSM with or without THI
216 treatment.

217 **Ameliorating effects of THI on DSS-induced colitis**

218 First, we confirmed that THI reduced peripheral lymphocytes from
219 2867.5±671.5 to 330.6±53.2 /μL under physiological condition (n=3 in each). DSS
220 administration induced diarrheal bloody stool, body weight loss, and increase in
221 the DAI score (Fig 1A). THI significantly decreased the DAI score induced by DSS.
222 The average length of removed colon on day 7 was significantly shorter in DSS
223 group than normal group, and THI improved the length of shortened colon
224 significantly (Fig 1B, 1C). Histological examination revealed that DSS-induced
225 colitis was severely inflamed, which was ameliorated by THI (Fig 1D). Assessment
226 of histological damage in each group according to Cooper's score showed that THI
227 significantly improved exacerbated histological score by DSS. The number of
228 lymphocytes infiltrating to inflamed mucosa was significantly decreased by THI
229 (Fig 1F). These results showed that THI ameliorated DSS-induced colitis clinically
230 and histologically.

231 **Suppression of DSS-induced cytokine expressions by THI**

232 Expressions of mRNA in all cytokines on day 7 were examined (Fig 2). DSS
233 increased mRNA expressions of IL-6, IFNγ, IL-17α, Nlrp3, TNFα and IL-1β in
234 colonic mucosa, which were significantly suppressed by THI.

235 **Altered distribution of systemic lymphocyte by THI**

236 Since THI reduced circulating and infiltrating lymphocyets, distribution of
237 lymphocytes with or without THI were examined by counting the total number of

238 $CD4^+$-cells in some organs, such as PPs, MLNs, spleen, thymus and BM (Table 1).
239 The results showed THI increased lymphocytes in BM, compatible with a
240 previous study.[25]

241 **FCM analysis of TDLs in rats with or without THI**

242 Since S1P agonist had ameliorating effects supposedly by suppressing naïve
243 lymphocyte egression from SLOs,[26] we investigated the mechanism how THI
244 affects on dynamics of naïve lymphocytes in PPs, a representative SLO in the gut.
245 First, we examined if THI changed the number of $CD62L^+$-T cells (naïve T cells) in
246 TDLs of naïve rats by FCM. We collected TDLs from naïve rats, and then TDLs
247 were gated by CD4 and expressions of CD62L were demonstrated (Fig 3A). We
248 confirmed that about 70% of TDLs expressed CD4, in which about 90% cells
249 expressed CD62L (data not shown). Compared with TDLs collected from control
250 animals, naïve T cells are decreased in TDLs collected from THI treated rats.
251 Next, we examined time-course changes in CD62L expression for 2 days in order to
252 evaluate how long THI affects the gut immunity. THI reduced the intensity but it
253 recovered slowly (Fig 3B). This FCM analysis showed THI decreased naïve T
254 lymphocytes in TDLs temporarily.

255 **Suppression of lymphocyte movement by THI and FTY720 in PPs under**
256 **microscopic observation**

257 Under physiological condition, injected lymphocytes attached to HEVs and
258 migrated across the endothelium into stroma, and then most of them moved within
259 stroma and some of them migrated to LCs during the microscopic observation
260 period (Fig 4A, Supporting information movie 1). LCs were visualized by Texas-red
261 in the lumen filled with unstained lymphocytes. In THI treatment group, almost
262 all of adhesive lymphocytes stayed still on the wall of HEVs after adhering during
263 the observation period, showing strong blockade of lymphocyte transendothelial

migration (TEM) into stroma (Fig 4B, Supporting information movie 2).

Next, we administered another S1P targeting drug, FTY720, to recipient rats before injecting untreated TDLs to clarify the effect on lymphocyte TEM. We expected that systemic administration of FTY720 did not show any effect on administered lymphocyte movement because the $S1P_1$ was supposed to be mainly expressed on the lymphocytes. But, surprisingly, most of the lymphocytes did not show TEM into stroma and stayed around HEVs after attaching to HEVs in the same way as THI treatment. Only a few of them showed TEM into stroma and moved around in a similar way with the physiological condition once they entered into stroma (Fig 4C, Supporting information movie 3). Average percentage of migrating lymphocytes in stroma in each group was shown in Fig 4D, implying that $S1P_1$ on HEVs was more strongly involved in S1P-induced blockade of lymphocytes from entering into PPs than that on lymphocytes. These results demonstrated that modification of lymphocyte migration by THI in PPs was induced in the entry of lymphocytes from HEVs into stroma at very early stage and leading to the reduction of naïve T cells in TDLs.

Comparison of ameliorating effects of THI and FTY720 on DSS-induced colitis

DSS-induced colitis was significantly ameliorated clinically and histologically by both FTY720 and THI (Figure 5). Although extent of amelioration was numerically greater in THI than FTY720, it was not significant. Inflammatory gene expression of inflamed mucosa showed comparable result. This agrees well with the results of our intravital observation.

No Direct Effect of THI on lymphocytes or endothelial cells

THI induced no change to MRα4 integrin expression (Control: 92.3±3.3 % vs THI 80 mg/mL: 92.7±3.1 %, p=0.94, data not shown in other concentration). Quantitative RT-PCR showed that expression of adhesion molecules stayed

290 unchanged against control mRNA levels in the cell monolayers with every dosage of
291 THI (data not shown).
292

293 *Discussion*

294 Several reports showed that S1P agonists, FTY720 or Ozanimod, caused rapid
295 lymphopenia and ameliorated intestinal inflammation with intensive
296 immunosuppressive effects.[7,11,12] THI was also reported to reduce the number of
297 circulating lymphocytes in a dose-dependent manner (50-200 mg/L).[16,19] THI
298 increases S1P abundance in lymphoid tissues more than 100-fold and causes the
299 S1P gradient to disappear.[13] Although previous reports of THI showed
300 immunomodulatory effects in the thymus,[27,28] its immunomodulatory property on
301 gut immunity has never been investigated. Therefore, we attempted to show the
302 ameliorative effects of this SPL inhibitor on murine colitis for the first time.

303 In this study, we evaluated the effects of modification of S1P by an SPL
304 inhibitor, THI on PPs. S1P has been reported to function at many points of
305 lymphocyte migration, possibly by forming an S1P gradient in the stroma,[13]
306 modifying the endothelial tight junctions,[14] or controlling RTM via cooperation with
307 chemokine receptors.[15] For the first time, we showed that THI almost blocked the
308 entrance of lymphocytes through the endothelium by using a CLSM, compatible
309 with FCM analysis showing reduced ratio of naïve T lymphocytes in TDLs. In
310 addition to the reduction by THI of circulating and colonic infiltrating lymphocytes,
311 our microscopic observation strongly suggested that the blockade of lymphocyte
312 entering from HEVs by THI are firmly involved in modulation of gut immunity,
313 possibly through modulating S1PR on HEVs, as shown in a previous report.[10] S1P
314 has been reported to strengthen endothelial junctions;[14] and can also sensitize
315 several chemokines necessary for migration, such as CCR7 and CXCR4.[29]
316 Additionally, the expressions of adhesion molecules were not altered by THI
317 compared with the control, implying that the mechanism is not due to modulation
318 of the expression of adhesion molecules on HEVs. Further study to clarify which

point is the most effective for S1P to control migration might lead to development of more specifically acting agents for IBD in the future.

Unlike the S1P agonist FTY720, THI increases S1P in PPs by reducing degradation (the S1P level is regulated by the balance of synthesis and degradation). Therefore, THI has certain differences compared with FTY720. First, the S1P gradient between the stroma and LCs will be offset by THI, which might lead to a strong blockade of lymphocyte flow. Moreover, THI has an agonistic effect on $S1P_2$, which works as a negative modulator of macrophage recruitment to inflamed mucosa on which FTY720 has no function.[30] This suggests that THI ameliorates inflammation via suppression of recruited macrophages. In addition, recovery from lymphopenia induced by THI takes less time than that from FTY720.[31] Taken together, there might be some advantages of THI over FTY720 in controlling S1P signaling.

As far as we know, there has been no report studying the effect of THI on inflamed areas. In chronic inflammation, lymphocytes are recruited through HEV-like vessels, which are morphologically identical to HEVs;[32,33] thus, it is also suggested that HEV-like vessels are associated with $S1P_1$. It is possible that THI modulates recruitment of lymphocytes by affecting microvessels in the inflamed mucosa. But we could not evaluate lymphocyte migration in colonic inflamed mucosa, because we were unable to focus on mucosal anatomy through the serosa by using a CLSM. Therefore, it is yet to be clarified how S1P is related to inflamed mucosa in the future. Additionally, in this study, we did not evaluate macrophage recruitment, but this might be one mechanism for ameliorating its effects and it should be clarified in future research.

Intravital observation found that THI suppressed naïve lymphocyte migration in the stroma of PPs, suggesting that blockade of naïve T lymphocyte entrance to

PPs might be one of the mechanisms for ameliorating colitis by THI. This study suggests that the SPL inhibitor THI might become a novel immunosuppressant for IBD therapy.

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448
449

482 **Table 1**

483 The number of lymphocytes in lymphoid organs after THI administration

484 ($x10^5$/mouse)

485

	Control	THI
Spleen	58.39±0.62	41.77±1.08
Thymus	11.77±0.95	13.52±0.70
MLNs	0.11±0.02	0.21±0.07
PPs	0.75±0.73	0.64±0.30
BM	9.47±1.34	14.3±0.56

486

487

Figure legends

Figrure 1

Ameliorating effect of THI on DSS-induced colitis.

(A) DAI in each group. (B) Representative macroscopic findings of colon removed from control, DSS and DSS+THI group. Arrows show distal end. (C) Average length of removed colon in each group. (D) Representative microscopic findings of the distal colon assessed by H&E staining in each group. (E) Assessment of histological damage in each group according to Cooper's score. (F) **The number of infiltrating lymphocytes in proportion with the length of muscularis mucosa per 100μm in paraffin section of the colon.** All statistical analyses were performed with Wilcoxon rank sum tests with unequal variance. Data are expressed mean ± SEM (n=6 in control, n=10 in other groups). N.S.: not significant, ＊: $P<0.05$, ＃: $P<0.01$.

Figure 2

THI suppression of DSS-induced gene expressions of pathogenic mediators.

Expression of mRNA levels of (A) IL-6, (B) IFNγ, (C) IL-17α, (D) Nlrp3, (E) TNFα and (F) IL-1ß. Relative quantity of each mRNA standardized to GAPDH is expressed. All statistical analyses were performed with Wilcoxon rank sum tests with unequal variance. Data are expressed mean ± SEM (n=6 in control, n=10 in other groups). ＊: $P<0.05$, ＃: $P<0.01$.

Figure 3

The number of $CD62L^+$ thoracic duct lymphocytes (TDLs) analyzed by flow cytometry (FCM).

(A) THI decreased the number of $CD62L^+CD4^+$ cells in TDLs. (B) The intensity of

476 $CD62L^+$ in $CD4^+$ TDLs recovered as time advances from day 0 **(immediately after**
477 **administration of THI)** to 2. In all measurements, $5x10^4$ cells were acquired by
478 FCM. Single-parameter histogram of $CD4^+$-cells was determined compared with
479 nonspecific binding of isotype control. Then, $CD62L^+$-cells out of $CD4^+$-cells were
480 expressed in single-parameter histogram and intensities were compared in some
481 groups.
482
483 **Figure 4**
484 Suppression of lymphocyte migration by THI and FTY720 in PPs under
485 microscopic observation.
486 Microscopic observation of PPs under physiological condition (A), THI treated
487 condition (B) and FTY720 treated condition (C). (A) Attached lymphocytes on high
488 endothelial venules (HEVs) emigrated to stroma and some of them migrated to
489 lymph capillaries. (B) THI administered to recipient induced lymphocytes to
490 adhere on the wall of HEVs. (C) FTY720 administered to recipient induced
491 lymphocytes to adhere on the wall of HEVs but less effective than THI. (D)
492 Average percentage of migration is shown in each group.
493 3 experiments were performed in each group and average was shown ± SEM. ＊:
494 $P<0.05$ v.s. Control, ＃: $P<0.05$ v.s. FTY720.
495
496 **Figure 5**
497 Comparison of ameliorating effect of THI on DSS-induced colitis.
498 (A) DAI in each group. (B) Representative macroscopic findings of colon removed
499 from control, DSS, DSS+FTY720 and DSS+THI group. Arrows show distal end. (C)
500 Average length of removed colon in each group. (D) Representative microscopic
501 findings of the distal colon assessed by H&E staining in each group. (E)

Assessment of histological damage in each group according to Cooper's score. (F) Assessment of infiltrating lymphocytes in colonic mucosa. All statistical analyses were performed with Wilcoxon rank sum tests with unequal variance. Data are expressed mean ± SEM (n=6 / group). N.S.: not significant, ＊: P<0.05, ＃: P<0.01.

Supporting information

Movie 1

Microscopic observation of PPs under physiological condition.

Lymph capillaries were visualized by Texas-red with intra-vessel unstained lymphocytes. Naïve lymphocytes attached to HEVs and then transendothelial migration into stroma was observed, and then all of them moved within stroma and some of them migrated to lymph capillaries.

Movie 2

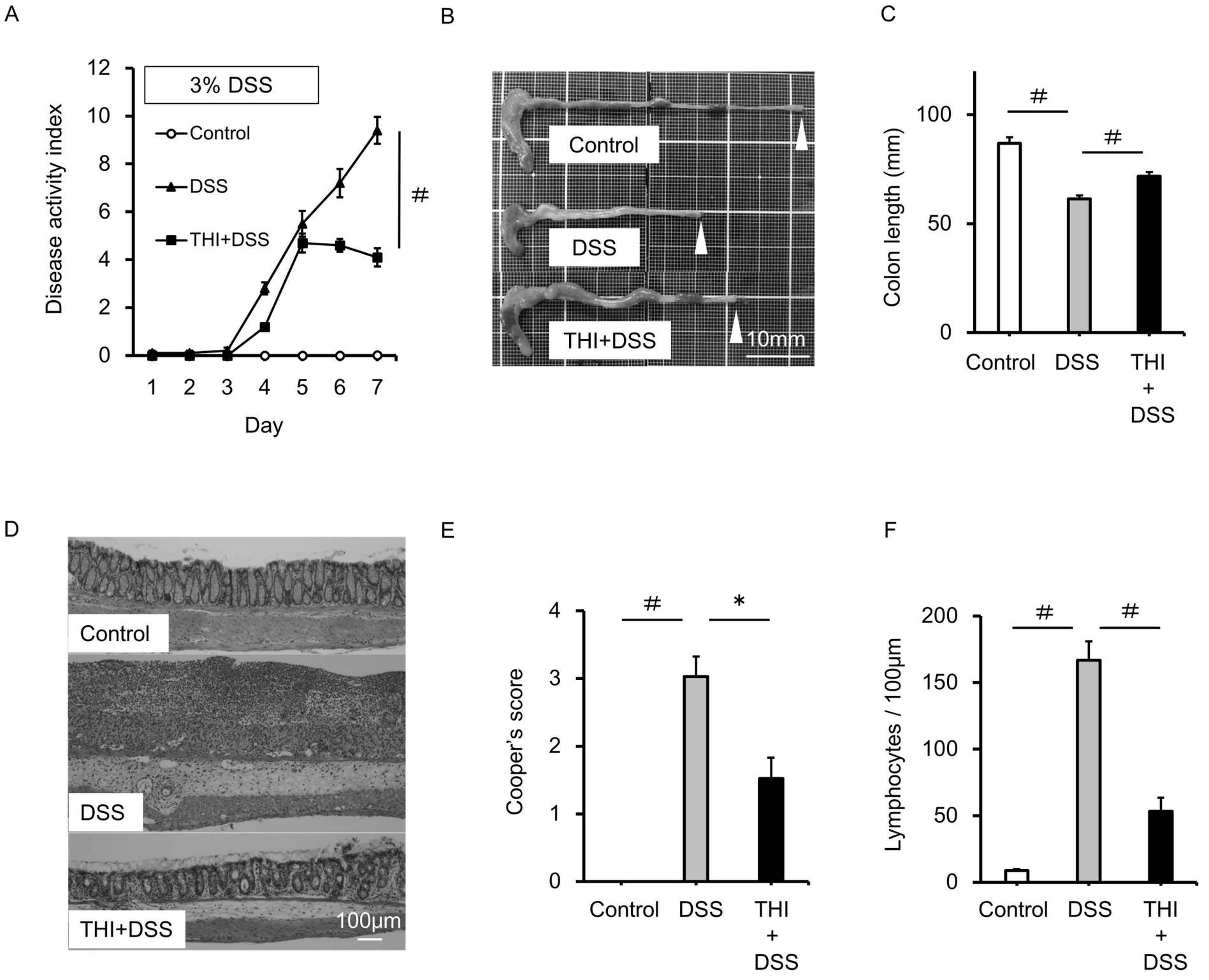
Microscopic observation of PPs under THI treatment.

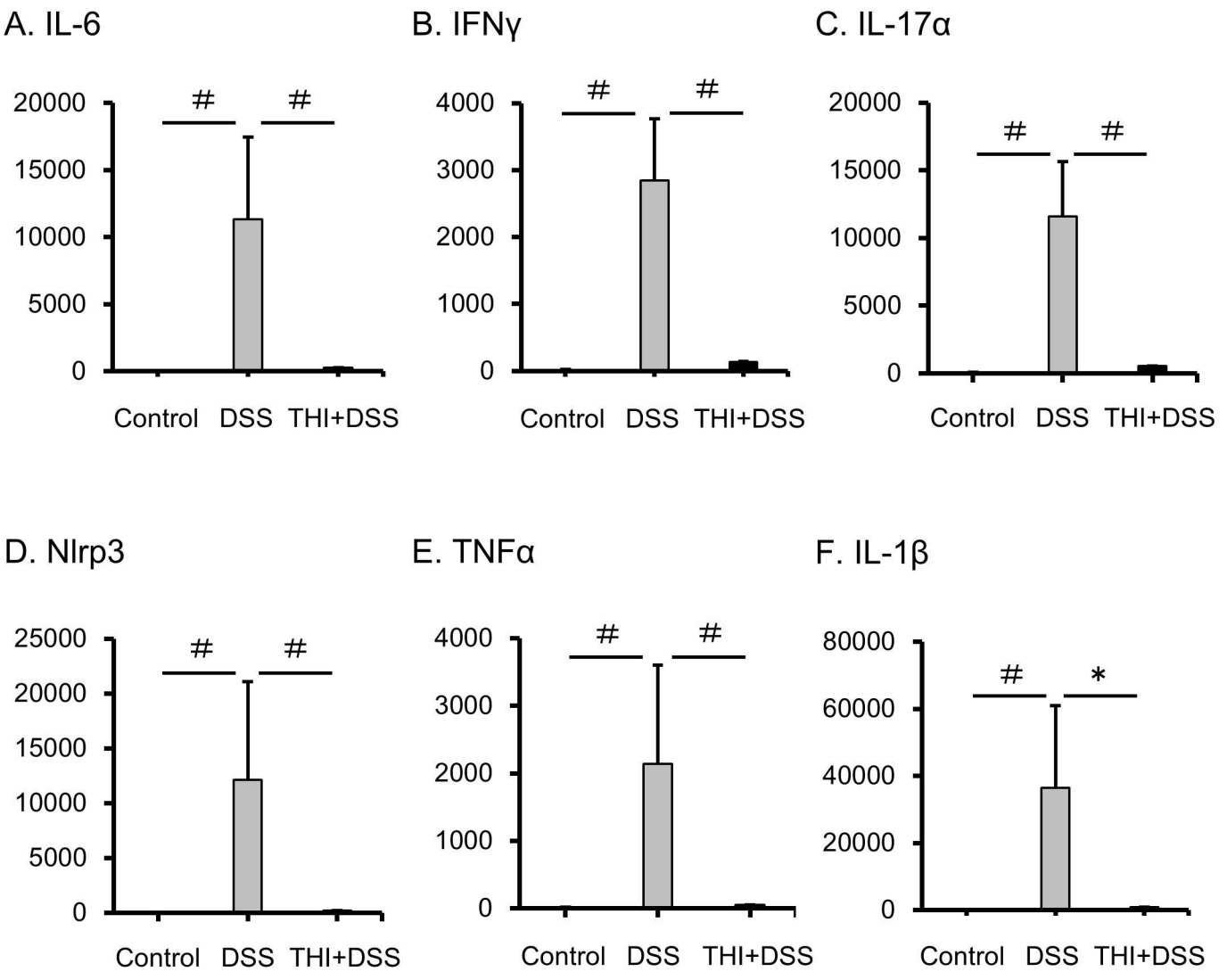
Almost all of adhesive lymphocytes stayed still on the wall of HEVs after adhering to HEVs, showing strong blockade of lymphocyte transendothelial migration into stroma.

Movie 3

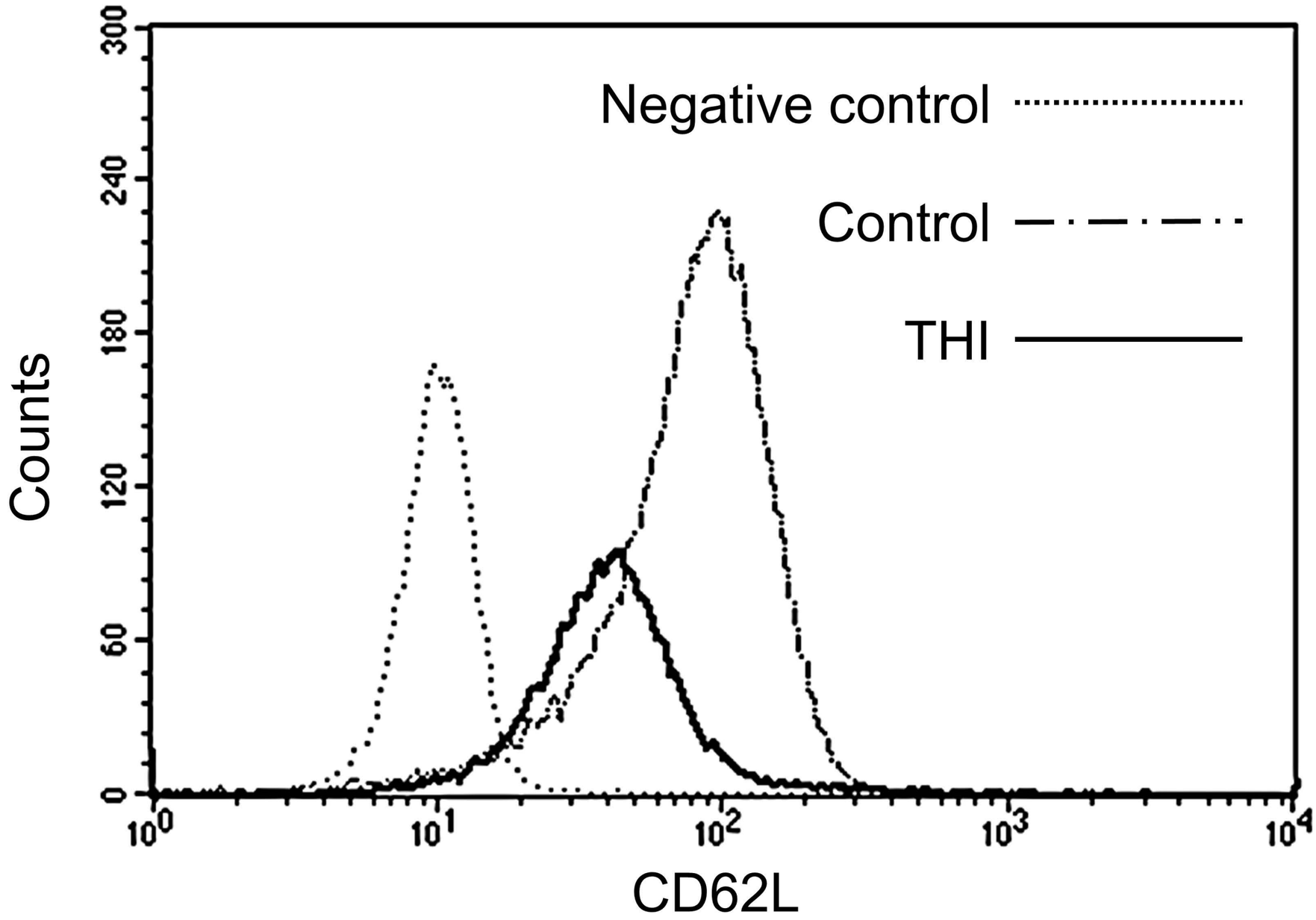
Microscopic observation of PPs under FTY720 treatment.

Lymphocytes attached to HEVs in the same way as control or THI treatment. But most of the lymphocytes did not show transendothelial migration into stroma and stayed around HEVs. Some of them showed transendothelial migration into stroma and moved around in a similar way with the physiological condition once they entered into stroma.

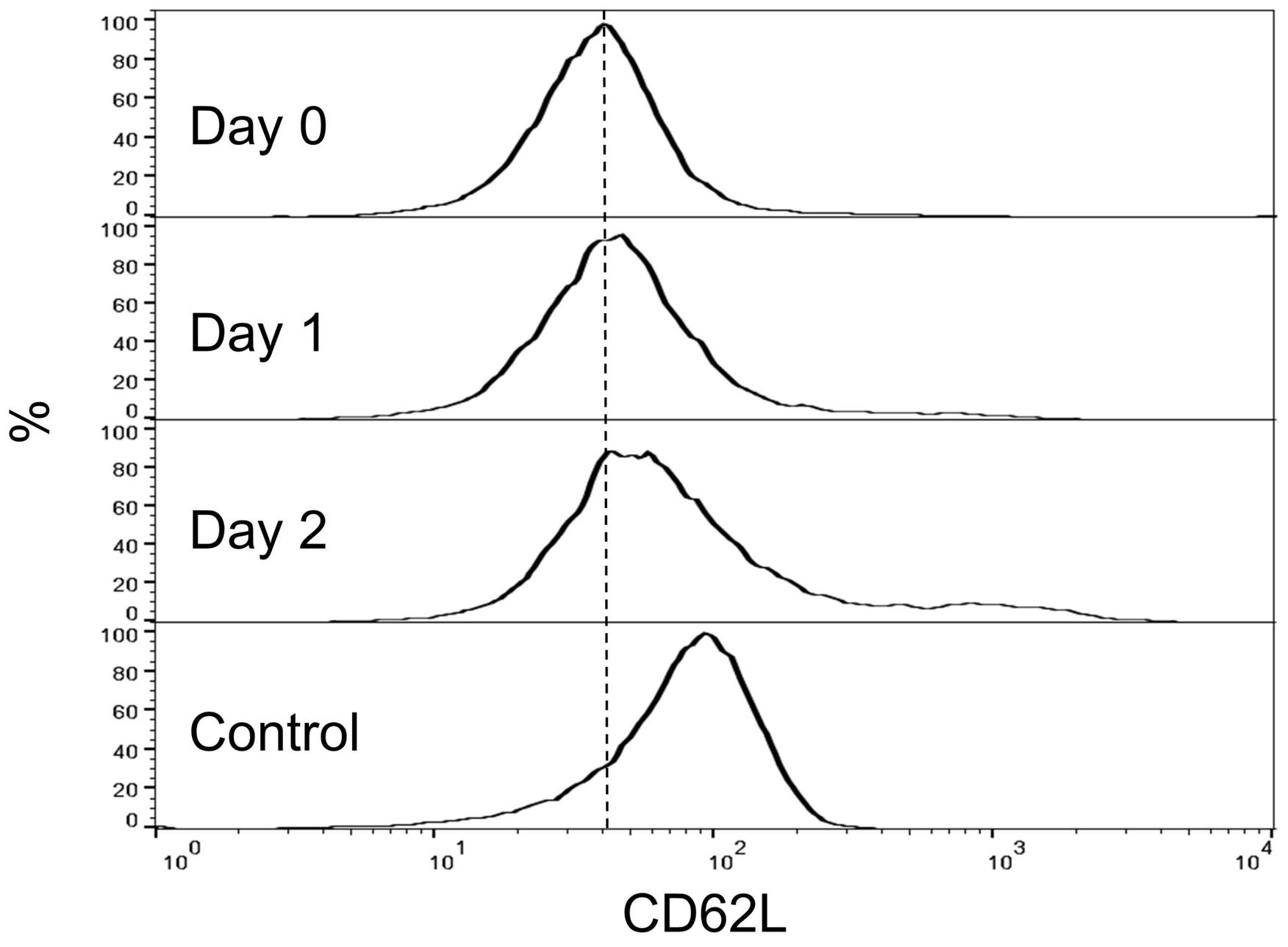

A
3% DSS
Control
DSS
THI+DSS
Disease activity index
Day
#
B
Control
DSS
THI+DSS
10mm
C
Colon length (mm)
Control
DSS
THI + DSS
D
Control
DSS
THI+DSS
100μm
E
Cooper's score
Control
DSS
THI + DSS
F
Lymphocytes / 100μm
Control
DSS
THI + DSS


A. IL-6
20000
15000
10000
5000
0
#
#
Control
DSS
THI+DSS
B. IFNγ
4000
3000
2000
1000
0
#
#
Control
DSS
THI+DSS
C. IL-17α
20000
15000
10000
5000
0
#
#
Control
DSS
THI+DSS
D. Nlrp3
25000
20000
15000
10000
5000
0
#
#
Control
DSS
THI+DSS
E. TNFα
4000
3000
2000
1000
0
#
#
Control
DSS
THI+DSS
F. IL-1β
80000
60000
40000
20000
0
#
*
Control
DSS
THI+DSS

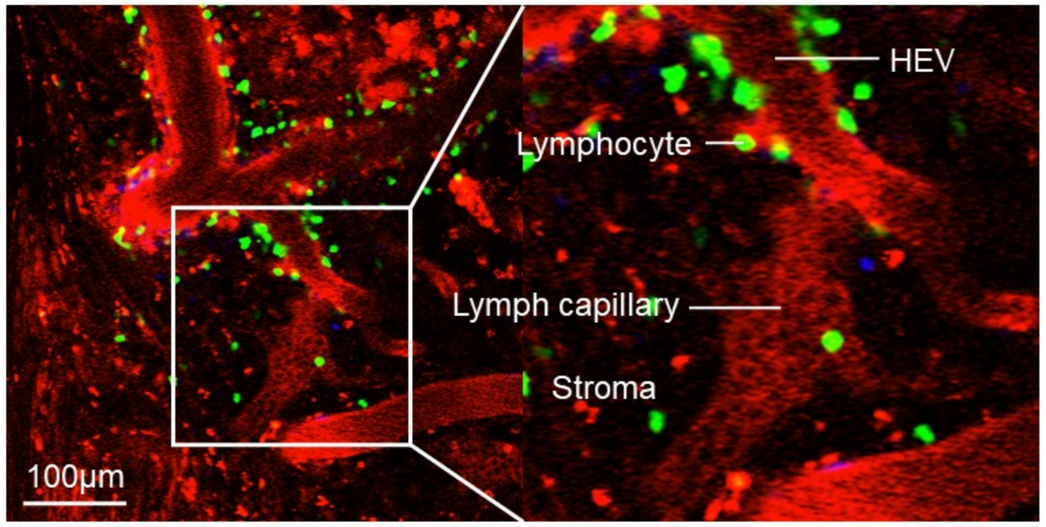
A


Negative control
Control
THI
Counts
300
240
180
120
60
0
10^0
10^1
10^2
10^3
10^4
CD62L

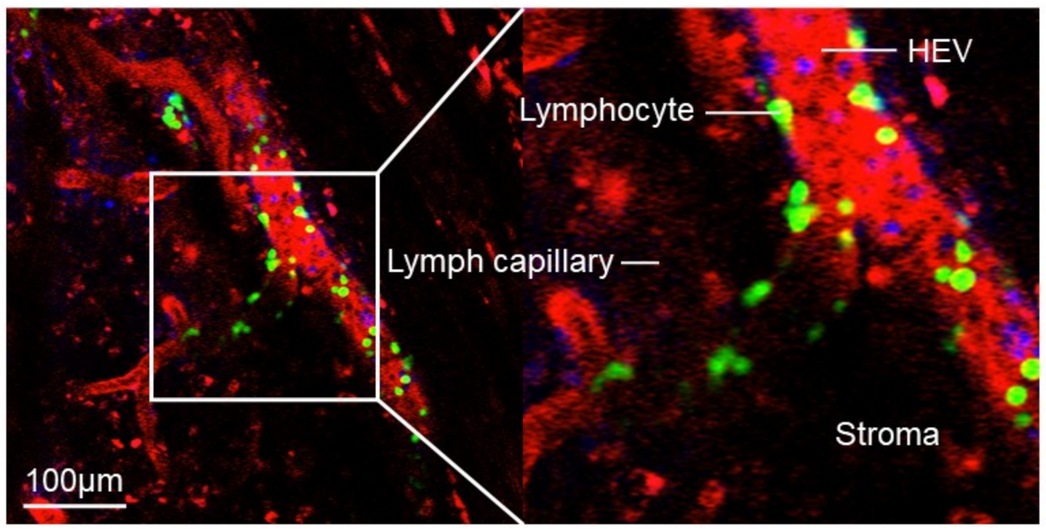
B


Day 0
Day 1
Day 2
Control
%
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
CD62L

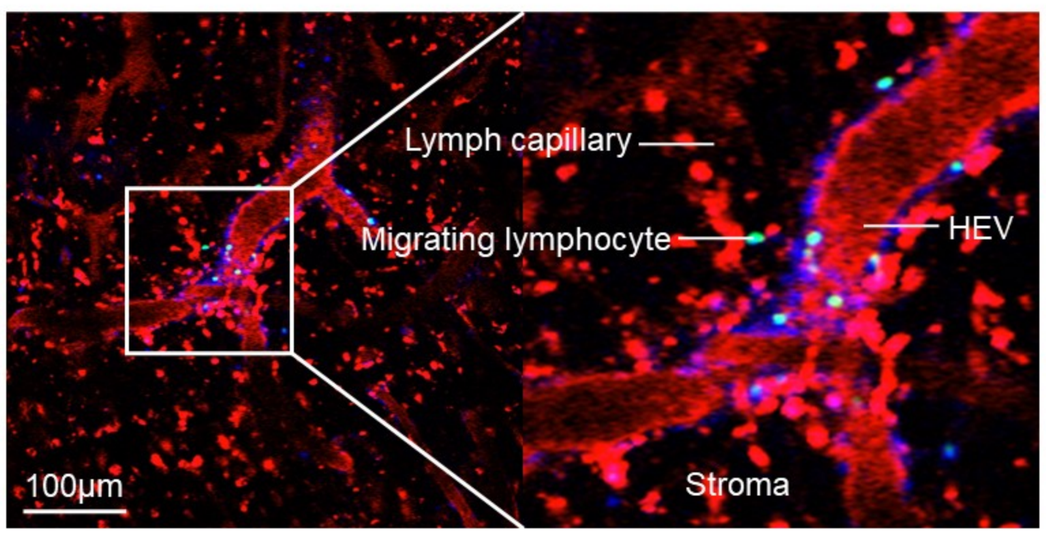
A


HEV
Lymphocyte
Lymph capillary
Stroma
100μm

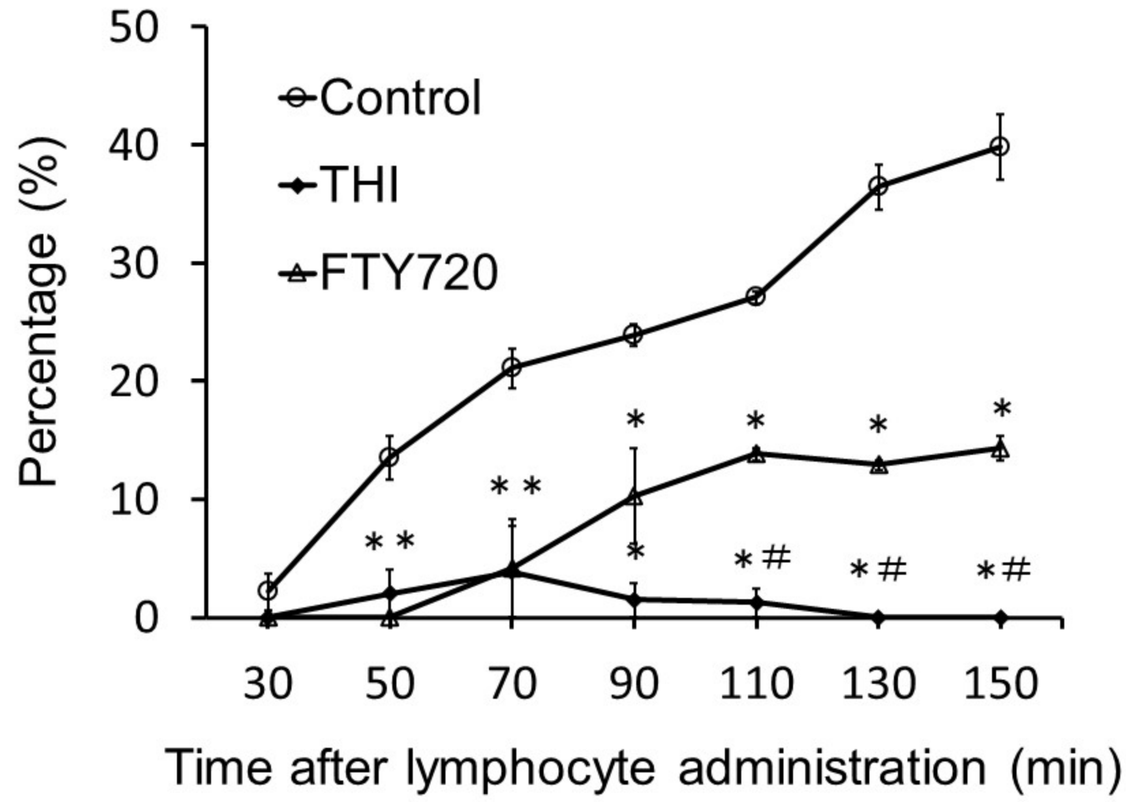
B

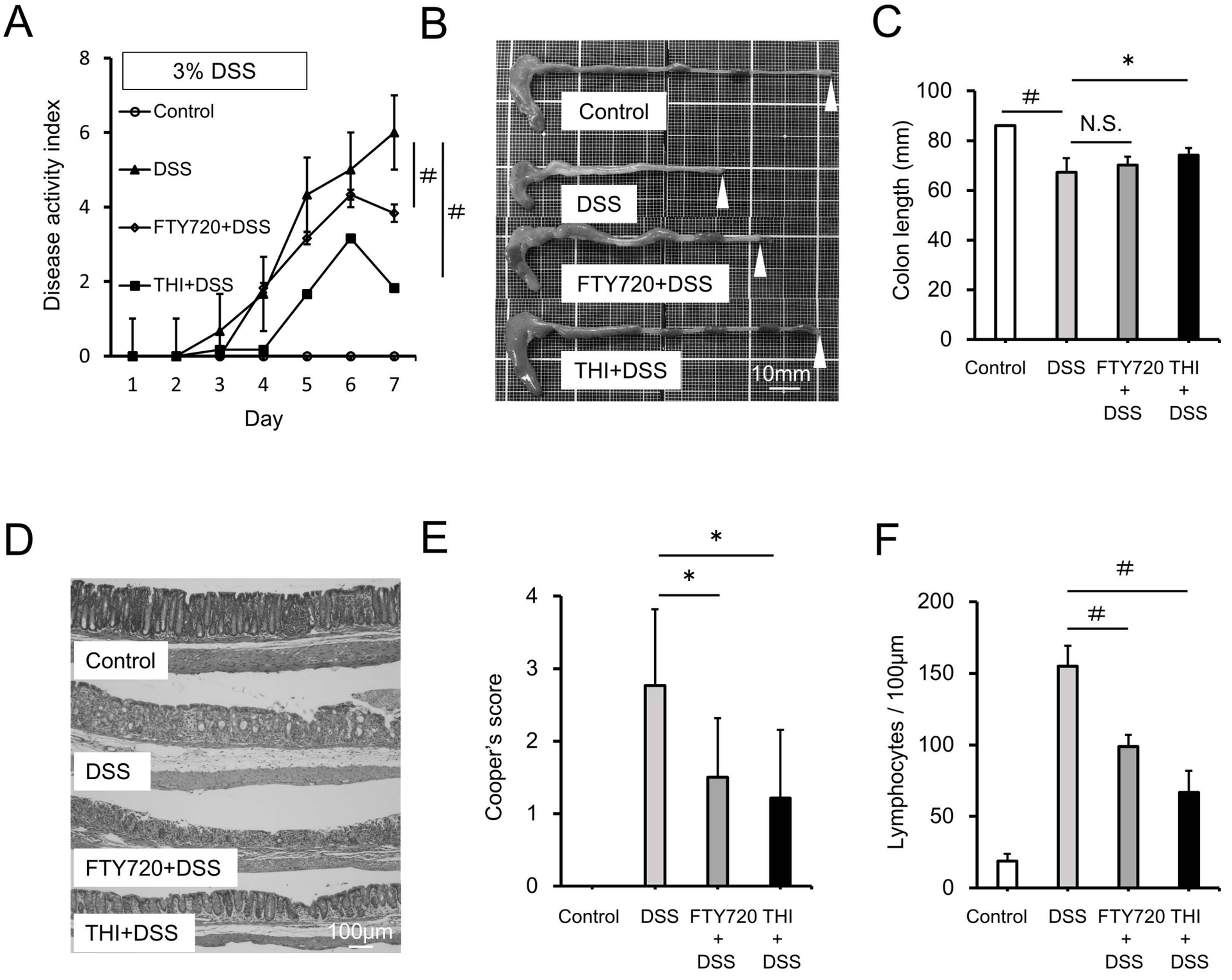

HEV
Lymphocyte
Lymph capillary
Stroma
100μm

C


Lymph capillary
Migrating lymphocyte
HEV
Stroma
100μm

D


Control
THI
FTY720
Percentage (%)
0
10
20
30
40
50
30
50
70
90
110
130
150
Time after lymphocyte administration (min)
* *
* *
*
*
*
*
*#
*#
*#


A
3% DSS
Control
DSS
FTY720+DSS
THI+DSS
Disease activity index
Day
#
#
B
Control
DSS
FTY720+DSS
THI+DSS
10mm
C
Colon length (mm)
#
*
N.S.
Control
DSS
FTY720 + DSS
THI + DSS
D
Control
DSS
FTY720+DSS
THI+DSS
100μm
E
Cooper's score
*
*
Control
DSS
FTY720 + DSS
THI + DSS
F
Lymphocytes / 100μm
#
#
Control
DSS
FTY720 + DSS
THI + DSS