METHODS

Tissue collection and thymocyte preparation

Postnatal thymus specimens were obtained from children undergoing corrective cardiac surgery at the UCLA Mattel Children's hospital. Fetal thymus specimens were obtained from the UCLA CFAR Gene and Cellular Therapy Core. Thymocytes were prepared and cultured at 4×10^7 cells/mL, as previously described,[E1] in serum-free medium (AT+dBSA) comprised of Iscove modified Dulbecco medium supplemented with 1100 μg/mL delipidated BSA (Sigma-Aldrich, St Louis, Mo), 85 μg/mL transferrin (Sigma-Aldrich), 2 mM L-glutamine, and 25 μg/mL penicillin/streptomycin.

Flow cytometry

Thymocytes were stained for 9 surface molecules (see the staining panel and antibody source in Tables E1 and E2). For S1P-R1 detection, cells were first stained with an unconjugated anti–S1P-R1 (or IgG_{2b} isotype control), followed by a phycoerythrin-conjugated anti-mouse IgG antibody. Then cells were stained for the other surface markers with the appropriate conjugated antibodies. Cells were washed with Nano Crystal buffer (eBioscience, San Diego, Calif) supplemented with 0.3% BSA (Sigma-Aldrich) and resuspended in Nano Crystal buffer before acquisition on a high-throughput LSRII cytometer (BD Biosciences, San Jose, Calif). Data were analyzed with FCS Express (De Novo Software, Glendale, Calif).

Cell sorting

Thymocytes were stained, as previously described,[E2] and sorted on a FACSAria cytometer (BD). A total of 10^8 thymocytes were stained for sorting in batches of 10^7 cells per tube at 10^7 cells/mL in AT+dBSA (see Table E1 for the antibody panel). After staining and washing in AT medium, cells were pooled and sorted on a FACSAria cytometer (BD). Cells were sorted on the basis of CD3 and CD69 expression by using CD27 and CD45RA to ensure the purity of populations. After sorting, cells were dissolved in TRI Reagent (TRIzol; Invitrogen, Carlsbad, Calif) for RNA isolation and subsequent PCR analysis, as previously described.[E2]

Quantitative real-time PCR

RNA was quantified by using Nanodrop, and TaqMan reverse transcription quantitative PCR was performed to determine the expression of the *S1PR1* to *S1PR5* and *KLF2* genes relative to that of the *GAPDH* internal control. Jurkat cells were used as positive controls and to generate standard curves for all S1P-R genes. Primer-probe conjugates were obtained from Invitrogen (Life Technologies/Fischer Scientific/Applied Biosystems) for *S1PR1* (assay ID: Hs01922614_s1), *S1PR2* (custom-designed assay using gene sequence from the National Center for Biotechnology Information), *S1PR3* (assay ID: Hs00245464_s1), *S1PR4* (assay ID: Hs02330084_s1), *S1PR5* (assay ID: Hs00928195_s1), and *KLF2* (assay ID: Hs00360439_g1). Target gene expression was normalized to primers amplifying *GAPDH* mRNA on an Applied Biosystems 7300 Real-Time PCR instrument. Data were analyzed in Microsoft Excel, and statistics were performed in GraphPad Prism 6 software (see below).

S1P chemotaxis/migration assay

Migration of thymocytes to S1P was assayed as described by Matloubian et al.[E3] Briefly, 1×10^7 cells (total thymocyte population) in serum-free medium (AT+dBSA) were loaded in the upper chamber and allowed to transmigrate for 3 hours at 37°C across 5-μm Transwell filters (Corning Costar) to S1P (Sigma) at the indicated concentrations with or without modulators (FTY720; Cayman Chemicals, Ann Arbor, Mich)/antagonists (W146, JTE-013; Cayman Chemicals) or in medium alone in the lower chamber. Cells were washed to remove modulators before loading into Transwell plate upper chambers. Input thymocytes and cells collected in the lower chamber were stained with mAbs to several surface markers for phenotyping of the migrated cells (see panel in Table E1). Migrated thymocytes were enumerated by collecting events for a fixed time (180 seconds) on a high-throughput LSRII cytometer. Cells from the input were diluted 1:20 and enumerated the same way to determine the percentage of transmigrating cells.

REFERENCES

E1. Gurney KB, Colantonio AD, Blom B, Spits H, Uittenbogaart CH. Endogenous IFN-alpha production by plasmacytoid dendritic cells exerts an antiviral effect on thymic HIV-1 infection. J Immunol 2004;173:7269-76.

E2. Colantonio AD, Epeldegui M, Jesiak M, Jachimowski L, Blom B, Uittenbogaart CH. IFN-alpha is constitutively expressed in the human thymus, but not in peripheral lymphoid organs. PLoS One 2011;6:e24252.

E3. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 2004;427:355-60.

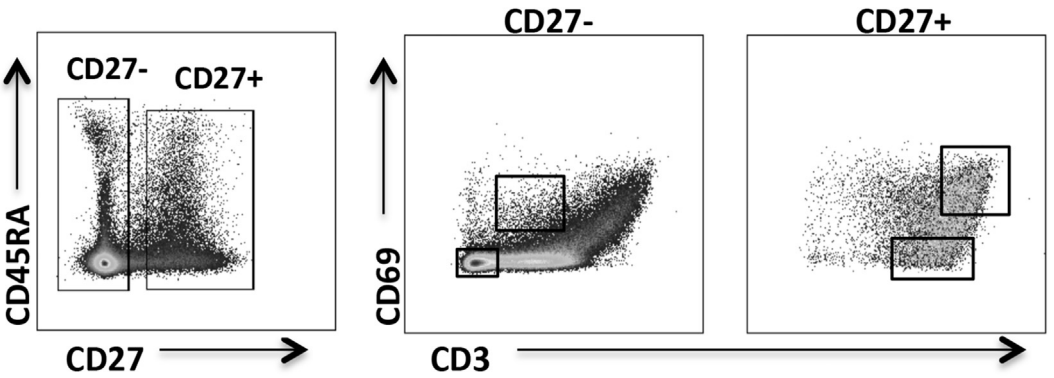

FIG E1. Gating schematic for CD3/CD69 thymocyte populations and profile of S1P-R1^{+} human thymocytes. Human postnatal thymocytes (age, 0 day to 44 years) were stained with antibodies to CD3, CD27, CD69, and CD45RA and gated as shown on CD27/CD45RA populations, followed by CD3/CD69 populations.

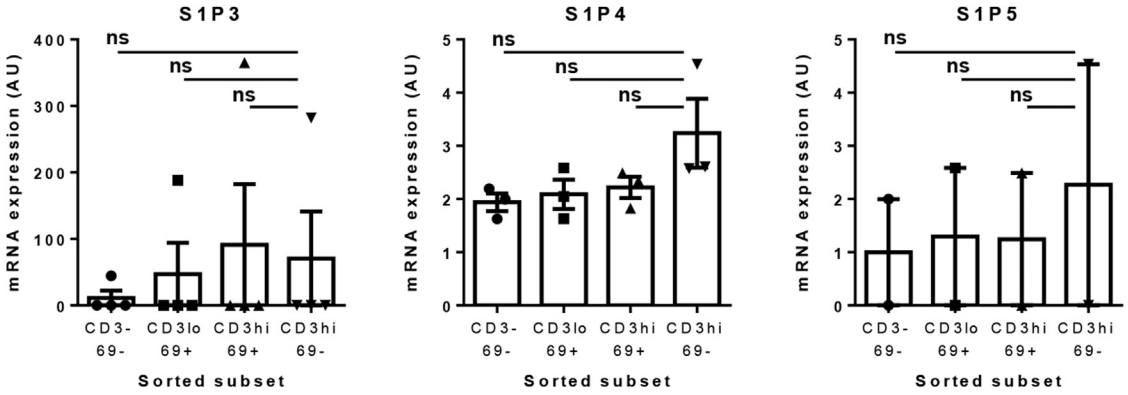

FIG E2. Expression of *S1PR3*, *S1PR4*, and *S1PR5* mRNA in CD3/CD69 thymocyte populations. Cell sorting was performed as described above to isolate CD3/CD69 populations. One-step RT-quantitative PCR was performed on mRNA isolated from these 4 populations to examine mRNA levels of *S1PR3*, *S1PR4*, and *S1PR5*. *ns*, Not significant.

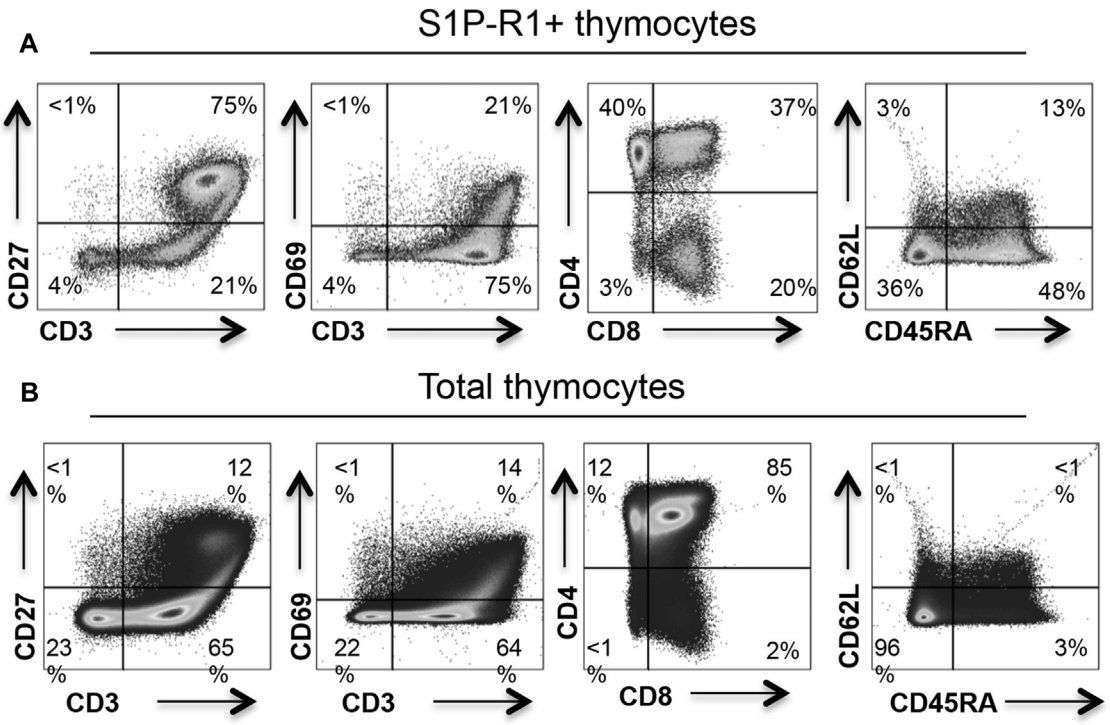

FIG E3. Phenotype of S1P-R1^{+} and total thymocytes. Expression of CD3, CD27, CD69, CD4, CD8, CD62L, and CD45RA on S1P-R1^{+} thymocytes (5 weeks old; **A**) compared with the total thymocyte population **(B)**.

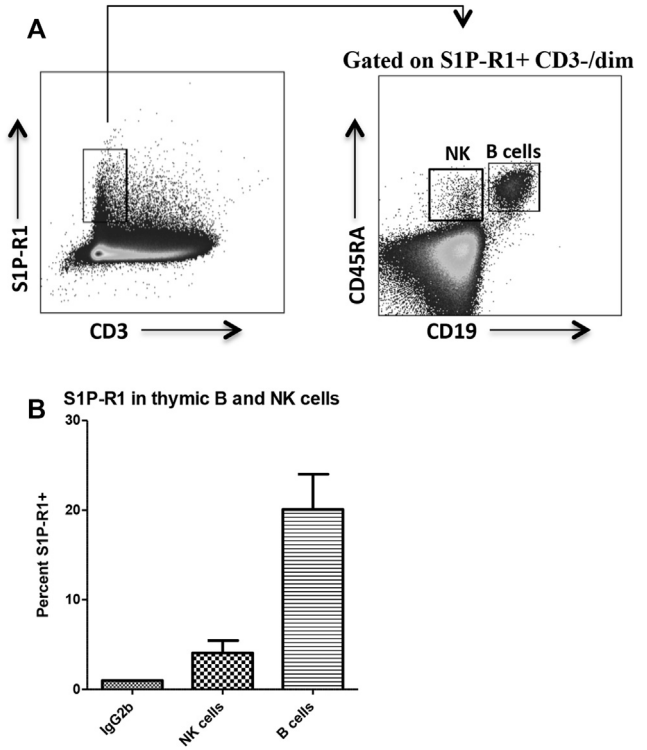

FIG E4. Thymic B and natural killer *(NK)* cells express S1P-R1. Human postnatal thymocytes were stained for expression of B and NK cell markers to ascertain whether these cells express S1P-R1. **A,** Expression of CD3, CD27, CD69, CD4, CD8, and CD45RA in total thymocytes (compared with S1P-R1^{+} thymocytes in Fig 3). **B,** Gating schematic for thymic B and NK cells. **C,** Expression of S1P-R1 in thymic B and NK cells relative to the IgG_{2b} isotype control.

TABLE E1. Antibody panels for flow cytometry and cell sorting

Experiment	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-eFluor 780	eFluor 450	eFluor 605^{NC}	eFluor 650^{NC}
Migration assay	CD69	CD31	CD45RA	CD4	CD27	CD8	CD25	CD62L	CD3
S1P-R1 staining	CD69	S1P-R1 or isotype + anti-Ms IgG	CD45RA	CD4	CD27	CD8	CD25	CD62L	CD3
Cell sorting	CD69	CD3	CD45RA	—	CD27	—	—	—	—

APC, Allophycocyanin; *eFluor 605^{NC}*, eFluor Nano Crystal; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.

TABLE E2. Antibody sources

Antibody	Manufacturer
CD3 eFluor 650^{NC}	eBioscience
CD3 PE	BD
CD4 PE-Cy7	eBioscience
CD8 APC-eFluor 780	eBioscience
CD25 eFluor 450	eBioscience
CD27 APC	BD
CD27 PE	BD
CD31 PE	eBioscience
CD45RA PerCP-Cy5.5	eBioscience
CD62L eFluor 605^{NC}	eBioscience
CD69 FITC	BD
S1P-R1 (unconjugated)	R&D
Mouse IgG2b isotype control	R&D
anti-Mouse IgG PE	eBioscience

APC, Allophycocyanin; *eFluor 605^{NC}*, eFluor 605 Nano Crystal; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.

TABLE E3. Summary of statistics data

Figure		Sample size (no.)	Test performed	*P* values	Summary
Fig 1	A	n = 10	Ratio-paired *t* test	.5673/.0758/.0029/.0001 (in figure order)	NS/NS/***/***
	B	n = 10	Two-way ANOVA	<.0001 for differences between populations and effect S1P	****
	C	Control: n = 10 FTY720: n = 8	Paired 1-way ANOVA followed by Dunnett multiple comparison test, each value compared with no S1P for each condition, with or without FTY720	1-way ANOVA: control, <.0001/FTY720, .0274	****/*
Fig 2	A	n = 4	One-way ANOVA followed by Dunnett multiple comparison test, each value compared with most mature subset ($CD3^{hi}CD69^{-}$)	ANOVA: <.0001	****
	B	n = 3		ANOVA: .0652	NS
	C	n = 4		ANOVA: .8230	NS
Fig 3	C	n = 18	One-way ANOVA, followed by Tukey multiple comparison test	ANOVA: <.0001	****
	D	n = 15	One-way ANOVA followed by Tukey multiple comparison test	ANOVA: <.0001	****
Fig 4	B	n = 5	Paired 2-tailed *t* test	.0043	**
Fig 5		n = 5	Paired 2-tailed *t* test	.0057 (S1P-R1)	**
		n = 4		.0101 (KLF2)	**
		n = 5		.3468 (IL-7R)	NS

NS, $P > .05$.
*$P < .5$.
**$P < .01$.
***$P < .001$.
****$P < .0001$.