The Journal of Immunology

Naive CD4$^+$ T Lymphocytes Circulate through Lymphoid Organs To Interact with Endogenous Antigens and Upregulate Their Function

This information is current as of October 17, 2021.

Michio Tomura, Kikuji Itoh and Osami Kanagawa

Supplementary Material	http://www.jimmunol.org/content/suppl/2010/03/18/jimmunol.0903946.DC1
References	This article **cites 40 articles**, 17 of which you can access for free at: http://www.jimmunol.org/content/184/9/4646.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription	Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription
Permissions	Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

Naive CD4⁺ T Lymphocytes Circulate through Lymphoid Organs To Interact with Endogenous Antigens and Upregulate Their Function

Michio Tomura,* Kikuji Itoh,† and Osami Kanagawa*

Naive T lymphocytes recirculate through the lymph-vascular system and enter and exit lymphoid organs. Using mice expressing the photoconvertible fluorescence protein Kaede, we demonstrated that naive T cells seek to interact with endogenous Ags after migrating to the lymphoid organs. The interaction with endogenous Ags transiently induces CD69 expression on T cells, which prolongs retention in the lymphoid organs. Cells that fail to express CD69 or lose CD69 expression migrate to other lymphoid organs. Functionally, CD69⁺-naive CD4⁺ T cells exhibit faster and greater cytokine production than do CD69⁻ naive CD4⁺ T cells. These results indicate that CD4⁺ T cells continuously migrate to interact with endogenous Ags, and such an interaction plays an important role in the Ag reactivity of naive CD4⁺ T cells. *The Journal of Immunology*, 2010, 184: 4646–4653.

Cells of hematopoietic origin migrate through the whole body to accomplish specific functions. Lymphocytes migrate continuously through secondary lymphoid organs and seldom migrate out to tissues in the steady state (1–5). However, upon infection with pathogens, a series of events take place to initiate immune response and to eliminate the pathogens. The initial phase of the response is mediated by the recruitment of cells of the innate immune system, such as neutrophils, macrophages, and dendritic cells, to the site of infection where they are activated by pathogens via pattern-recognition receptors (6). The activation of the cells by pathogens induces the expression of a variety of proteins necessary for the initial control of the pathogens and the stimulation of adaptive immune response. Activated APCs then migrate to lymphoid organs where they interact with T and B lymphocytes (6, 7). This step is critical for the generation of an Ag-specific adaptive immune response.

The interaction between innate and adaptive immune systems during infection in vivo is well coordinated by a complex series of events involving cell migration to and retention at different anatomical sites (8–10). One of the molecules involved in cell migration in vivo is CD69. The CD69 molecule is a type I C-type lectin expressed by activated T cells (11–13). CD69 is expressed on Ag-activated T cells, some T cells during homeostatic expansion in vivo, and positively selected CD4⁺ and CD8⁺ double-positive thymocytes (14). In all cases, the expression of CD69 by the cells is a transient event. It was shown that the downregulation of CD69 plays a critical role in the emigration of mature thy-

mocytes to secondary lymphoid organs (15). In the periphery, the expression of CD69 molecules downregulates S1P1, leading to the inhibition of lymphocyte egress from the lymph node (LN) and the retention of lymphocytes in the LN (16–18). These results clearly demonstrate the mechanisms by which Ag-activated T cells accumulate in the draining LNs via interaction with cognate Ag-bearing dendritic cells for the induction of CD69 and the subsequent downregulation of S1P1. Recently, the molecular mechanisms of T cell retention within and egress from LNs were reported. Lymphocyte egress from LNs is promoted by S1P1 to overcome retention signals mediated by CCR7 and additional Gαi-coupled receptors (19). However, the biological significance of naive T cell retention in lymphoid organs and recirculation in the steady state in the maintenance of immune homeostasis is unclear.

Recently, we reported a novel system for monitoring cell migration in vivo by using photoconvertible protein Kaede transgenic (Tg) mice. This system allowed us to demonstrate that CD4⁺, CD8⁺, and B lymphocytes have distinct migration kinetics in vivo in the steady state (20). In this study, using Kaede-Tg mice, we analyzed the migration of CD4⁺ T cells in vivo in the context of their phenotypic and functional changes. We demonstrate that CD69 expression on CD4⁺ T cells is regulated by the interaction with endogenous Ags via surface Ag receptors and that naive and memory CD4⁺ T cells show clear differences in their capacity to interact with Ags in vivo. Naive and memory CD4⁺ T cells that fail to interact with Ags rapidly emigrate from the LN and move to other LNs to seek an opportunity to interact with the Ags. Cells that successfully interact with the Ags and express CD69 show prolonged retention in the LN. However, the expression of CD69 is transient, and the loss of CD69 expression results in emigration from the LN. Thus, CD4⁺ T cells are continuously migrating through lymphoid organs to seek endogenous Ags for interaction. This interaction transiently retains the cells in the local LNs and regulates cell migration and emigration. Furthermore, functional analysis of naive CD4⁺ T cells revealed that cells expressing CD69 exhibited significantly rapid and strong responses to the stimuli through TCR compared with the CD69⁻ population. These results suggest that the interaction between naive CD4⁺ T cells and endogenous Ags enhances the functional activity of the T cells, which

may play an important role in preparing the adaptive immune system to deal with pathogens expressing cognate Ags to T cells.

Materials and Methods

Mice

Kaede-Tg mice (BDF1mixed background) (20), backcrossed to C57BL/6 mice for more than eight generations, were used for experiments. OVA-specific DO11.10 TCR-Tg Rag2$^{-/-}$ mice were generated by crossing DO11.10 TCR-Tg mice (21) (a kind gift from Dr. Kenneth M. Murphy, Washington University School of Medicine, St. Louis. MO) with BALB/c Rag2$^{-/-}$ mice (purchased from Taconic Farms, Germantown, NY). The Tg mice expressing pigeon cytochrome c-specific and I-E^k–restricted 2B4 TCR (22) with a Rag2$^{-/-}$ background were described previously (23). MHC class II knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (24). C57BL/6 and BALB/c mice were purchased from CREA Japan (Tokyo, Japan). The mice were maintained under specific pathogen-free (SPF) conditions, and experiments were carried out in compliance with institutional guidelines.

Cell transfer

Cells from peripheral LNs (PLNs) and spleen were transferred i.v. to recipient mice. In some experiments, cells were labeled with 10 μM CFSE for 10 min before the transfer. For the transfer of CD69$^+$ and CD69$^-$ T cells, LN cells were stained with biotin–anti-CD69 mAb, followed by anti-biotin MACS beads. Then, CD69$^+$ and CD69$^-$ cells were sorted by MACS (Miltenyi Biotec, Auburn, CA) before transfer. For experiments using CD4$^+$ T cell transfer to wild type (WT) and MHC class II KO mice, LN and spleen cells of C57BL/6 mice were stained with an Ab mix provided in the Dynal Mouse CD4 Cell Negative Isolation Kit (Invitrogen, Oslo, Norway) and rat anti-MHC class II mAb (M5/114.15.2, BD Biosciences, San Jose, CA). Then, the Ab-stained cells were depleted with anti-rat Ig beads in accordance with the protocol provided by the manufacturer. CD11c$^+$ cells were depleted from purified cells by staining with anti-CD11c, followed by anti-allophycocyanin MACS beads. Ten million purified CD4$^+$ cells (class II$^+$ cells and CD11c$^+$ cells were not detected, and the purity of CD4$^+$ cells was >98%) were transferred to WT and MHC KO mice.

Flow cytometry analysis

Cells were treated with 2.4G2 hybridoma culture supernatant to block Fc binding and were stained with FITC-, PE-, PE-Cy7–, allophycocyanin-, or allophycocyanin-Cy7–conjugated anti-mouse CD4, anti-mouse CD25, anti-mouse CD44, anti-mouse CD62L, or KJ1-26. CCR7 staining was carried out using biotinylated anti-CCR7 and allophycocyanin-conjugated streptavidin. CD69 expression was determined using biotin-conjugated anti-CD69 mAb, followed by Pacific blue (Invitrogen) or Alexa 633-conjugated streptavidin. 7-Aminoactinomycin D was added to the sample to gate out dead cells. These reagents were purchased from BD Pharmingen (San Diego, CA), eBioscience, or BioLegend. Intracellular Foxp3 was stained with an Anti-mouse Foxp3 Staining Kit (eBioscience, San Diego, CA). Stained samples were analyzed on FACSCalibur (BD Pharmingen) or JSAN (Bay Bioscience, Kobe, Japan). Kaede green and red signals were detected with FITC (green) and PE (red) channels, respectively. Flow cytometry data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Lymph collection

Mice were anesthetized and positioned on their right side. The abdomen was opened through a left subcostal incision, and the cisterna chyli was identified as a small cream-colored sac located dorsal to the left renal LN. A 30-gauge needle was inserted into the cisterna chyli, and lymph fluid was harvested.

Photoconversion

Photoconversion of inguinal LN was carried out with a method described previously (20). Briefly, Kaede-Tg mice were anesthetized, and the inguinal LN was visualized through an incision made on the abdominal skin. After covering the surrounding tissue with aluminum foil, the LN was exposed to violet light (95 mW/cm^2 with a 436-nm band pass filter with Spot UV curing equipment [SP500], Usio, Tokyo, Japan) with continuous instillation of warmed PBS. The wound was closed with sutures.

Cytokine production

CD4$^+$ T cells were purified from the LN cells of C57BL/6 mice with a Dynal Mouse CD4 Negative Isolation Kit (Invitrogen). Purified CD4$^+$ T cells were stained with anti-CD4, anti-CD25, anti-CD62L, and anti-

CD69 Abs, as described above. CD69$^+$CD25$^-$CD62L$^+$CD44^{midlow}CD4$^+$ and CD69$^-$CD25$^-$CD62L$^+$CD44^{midlow}CD4$^+$ cells were sorted by JSAN (Bay Bioscience) (purity of each population was >95%). Purified cells (5 × 10^4 or 5 × 10^5 cells) were stimulated with plate-coated anti-CD3 mAb (2C11, 10 μg/ml) in a final volume of 200 μl DMEM containing 5% FCS in flat-bottom microtiter plates, and the supernatant was harvested at indicated times. Cytokine concentrations in the supernatants were measured with a Cytometric Bead Array system (BD Bioscience).

Results

Induction of CD69 expression on TCR-Tg naive CD4$^+$ T cells by endogenous Ags

OVA-specific CD4$^+$ T cells from DO11.10 TCR-Tg Rag2$^{-/-}$ mice (21) showed no signs of activation in vivo and expressed a high level of CD62L and medium to low levels of CD44 (Supplemental Fig. 1). However, a small, but significant, percentage of cells expressed CD69, which is known to be an early activation marker of T cells (11, 12) (Fig. 1A). These DO11.10 T cells were labeled with CFSE and cotransferred with the same number of CFSE-labeled WT CD4$^+$ T cells into normal BALB/c mice. Four days after the transfer, the cells were analyzed for cell-cycle progression and the expression of CD69. DO11.10 and WT CD4$^+$ T cells did not show significant proliferation in normal BALB/c mice, as reported previously (25). However, a large fraction of DO11.10 T cells (>90%) expressed CD69 when small numbers (1 × 10^4 and 1 × 10^5 cells) of cells were transferred. The percentage of CD69$^+$ cells decreased to ~60% when a large number of cells (1 × 10^6 cells) was transferred. The percentage of WT CD4$^+$ T cells expressing CD69 was stable, regardless of the number of cells used for the transfer (Fig. 1B). Thus, these results suggest that the induction of CD69 expression is a unique feature of DO11.10 T cells and is not a result of the cell-transfer experiment.

CD69 is known as an early marker of T cell activation by cognate Ag stimulation (11, 12), and the cell number-dependent decrease in CD69$^+$ DO11.10 T cells after the transfer prompted us to test the possibility of competition among DO11.10 T cells for the interaction with endogenous Ags. To this end, 1 million CFSE-labeled DO11.10 T cells were transferred into normal BALB/c mice, either alone or with an excess number (1 × 10^7 cells) of nonlabeled DO11.10 cells as competitors. Four days after the transfer, the expression of CD69 on the CFSE-labeled DO11.10 T cells was analyzed. More than 60% of transferred DO11.10 T cells expressed CD69 in the absence of a competitor, similar to the result in Fig. 1B. The percentage of CD69$^+$ cells decreased to 19% in the presence of a large number of nonlabeled DO11.10 competitor cells (Fig. 1C). In contrast, the presence of 1 × 10^7 WT T cells did not affect the CD69 expression of CFSE-labeled DO11.10 T cells, suggesting that the inhibition of CD69 expression occurred in a DO11.10 T cell-specific manner. Nonlabeled DO11.10 T competitor cells expressed the same percentage of CD69 as CFSE-labeled DO11.10 T cells (19%). Thus, the results suggest that the induction of CD69 expression on transferred DO11.10 T cells is a result of the interaction with endogenous Ags. However, the available Ags (either the number of cells expressing endogenous Ags or the available niche for the interaction) are limited; thus, DO11.10 T cells compete with each other for interaction with the Ags to express CD69.

Pigeon cytochrome c-specific CD4$^+$ T cells from 2B4 TCR-Tg Rag2$^{-/-}$ mice were tested in the same manner as B10.BR recipient mice. When 1 × 10^5 2B4 T cells were transferred, 21% of the cells became positive for CD69 expression, which was significantly greater than the percentage of CD69$^+$ T cells (5%) prior to the transfer (Supplemental Fig. 2). When the number of transferred T cells was increased (1 × 10^6 cells), the percentage of CD69$^+$ 2B4 T cells decreased to 6.2%, under identical conditions.
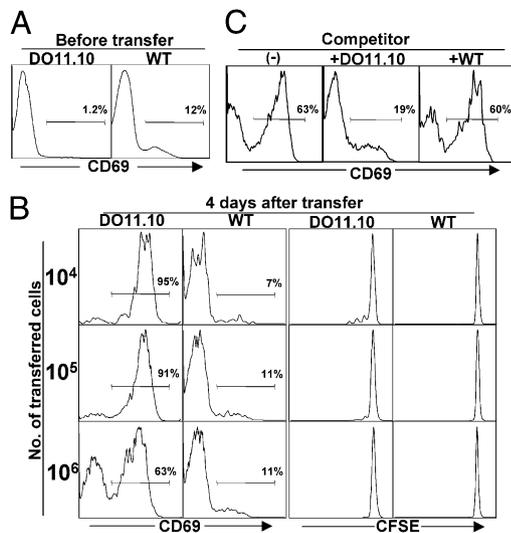
FIGURE 1. Induction of CD69 expression and cell-cycle progression of transferred DO11.10 T cells after transfer. *A*, LN cells from DO11.10 TCR-Tg Rag2$^{-/-}$ mouse or BALB/c mice (WT) were stained with anti-CD4, KJ1-26, and anti-CD69 mAbs and subjected to flow cytometry. CD69 expression of CD4$^+$KJ1-26$^+$ cells from DO11.10 TCR-Tg Rag2$^{-/-}$ mice and CD4$^+$ T cells from WT mice is shown. *B*, Indicated numbers of CFSE-labeled DO11.10 T cells from DO11.10 TCR-Tg Rag2$^{-/-}$ mouse and CD4$^+$ T cells from BALB/c WT mice were transferred to BALB/c WT mice i.v. Four days after the transfer, cells in the LNs (pool of cervical, brachial, axillary, inguinal, and popliteal LNs) were stained in the same manner as in *A*. CD69 expression and CFSE dilution of transferred CD4$^+$ KJ1-26$^+$ cells and CD4$^+$ T cells from WT mice are shown. Three animals for each group were independently analyzed with similar results, and the results of one representative analysis are shown. *C*, Intraclonal competition for the expression of CD69 in transferred DO11.10 T cells. CFSE-labeled DO11.10 T cells (1 × 10^6 cells) from DO11.10 TCR-Tg Rag2$^{-/-}$ mice were transferred with nonlabeled DO11.10 T cells (1 × 10^7 cells) (+DO11.10), nonlabeled CD4$^+$ T cells from BALB/c WT mice (1 × 10^7 cells) (+WT), or no competitor cells (−) to BALB/c WT mice i.v. Four days after the transfer, CD69 expression of transferred DO11.10 T cells and CD4$^+$ T cells of WT mice in the LNs was analyzed in the same manner as in *B*. CD69 expression of CFSE-labeled DO11.10 T cells is shown. Three animals for each group were independently analyzed with similar results, and the results of one representative analysis are shown.

These results demonstrate that in DO11.10 and 2B4 T cells, the interaction with endogenous Ags induces CD69 expression, but the available Ags may differ for DO11.10 T cells in BALB/c mice and 2B4 T cells in B10.BR mice.

CD69 expression in normal CD4$^+$ T cells

CD69 was expressed in a small fraction of CD4$^+$ T cells from LN and spleen, and very few, if any, CD4$^+$ T cells in circulation in peripheral blood and lymph expressed CD69 (Fig. 2*A*). The CD69$^+$ CD4$^+$ T cell fraction in LN contained more CD62L$^{negative/low}$, CD44^{high}, CD25$^+$, CCR7^{low}, and Foxp3^{low} cells than did the CD69$^-$ cell fraction (Supplemental Fig. 3). These results indicate that CD69$^+$ CD4$^+$ T cells contain more memory and regulatory T cell populations. However, a significant number of CD25$^-$CD62L^{high}CD44^{mid} naive CD4$^+$ T cells also expressed CD69 (Fig. 2*B*). Thus, CD69 expression cannot act as a marker for any particular subpopulation of CD4$^+$ T cells.

We then analyzed whether the heterogeneity of CD4$^+$ T cells in the expression of CD69 represents subpopulations of CD4$^+$ T cells that are capable of interacting with endogenous Ags, as demonstrated by T cells from TCR-Tg mice. CD4$^+$ T cells from LN cells were sorted into CD69$^+$ and CD69$^-$ populations, labeled with CFSE, and transferred to normal C57BL/6 mice i.v. Four days
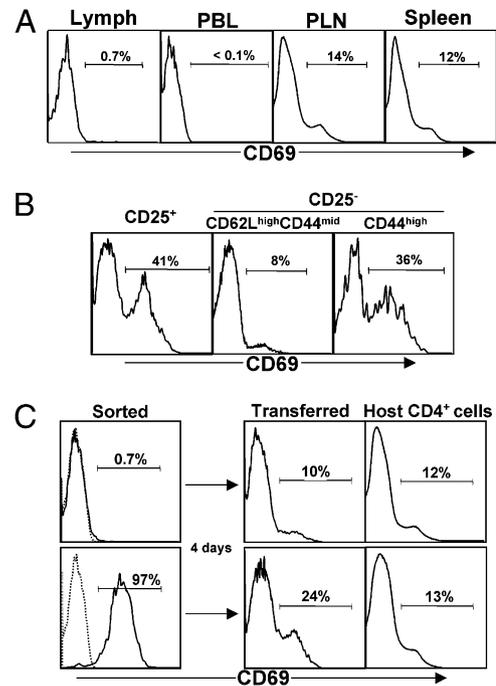

FIGURE 2. CD69 expression on CD4$^+$ T cells from normal B6 mice. *A*, Lymphocytes in lymph, peripheral blood, PLNs (pool of cervical, brachial, axillary, inguinal, and popliteal LNs), and spleen were stained with anti-CD4 and anti-CD69 and subjected to flow cytometry. *B*, Lymphocytes of PLNs in C57BL/6 WT mice were stained with anti-CD4, anti-CD25, anti-CD62L, anti-CD44, and anti-CD69 mAbs. CD4$^+$ T cells were separated into three groups based on the expression of CD25, CD62L, and CD44, and the expression of CD69 was analyzed for each population. *C*, Unstable expression of CD69 molecule on CD4$^+$ T cells after transfer. LN cells from C57BL/6 mice were stained with anti-CD4 and anti-CD69 mAbs, and CD69$^+$ and CD69$^-$ cells were sorted as described in *Materials and Methods*. Sorted CD69$^+$ and CD69$^-$ CD4$^+$ cells (1 × 10^6 cells) were labeled with CFSE and transferred to C57BL/6 WT mice i.v. Four days after the transfer, CD69 expression of transferred and host CD4$^+$ cells in the PLNs was analyzed in the same manner as in *A*. Three animals were independently analyzed for each group, with similar results, and the results of one representative analysis are shown.

after the transfer, in the mice that received CD69$^-$ CD4$^+$ T cells, 10% of the transferred CD4$^+$ T cells were positive for CD69, which is the same level as that of the host CD4$^+$ T cell population (12%). In the mice that received CD69$^+$ CD4$^+$ T cells, a large fraction of T cells lost the expression of CD69, and 24% remained positive for CD69 expression (Fig. 2*C*). These results clearly indicate that the expression of CD69 on CD4$^+$ T cells in vivo under nonimmunized conditions is unstable, similar to the transient expression of CD69 in Ag-activated T cells.

Migration of CD4$^+$ T cells and regulation of CD69 expression

The total lack of CD69$^+$ CD4$^+$ T cells in the circulation is fully compatible with the notion that CD69 regulates lymphocyte emigration from the LN (11, 12), and only CD69$^-$ T cells emigrate from the LN. Using a recently established mouse line expressing the photoconvertible fluorescence protein Kaede (20), we investigated the relationship between cell migration and the expression of CD69 in vivo, with minimum manipulation of the mice. Inguinal LNs were exposed to violet light, which caused all cells in the inguinal LNs to express red Kaede protein (20) (Fig. 3*A*). Cells in the photoconverted inguinal LNs and connecting axillary LNs were analyzed 24 h after the photoconversion. In this analysis, nonphotoconverted cells in the inguinal LNs are recent migrants from other lymphoid tissues, and photoconverted cells
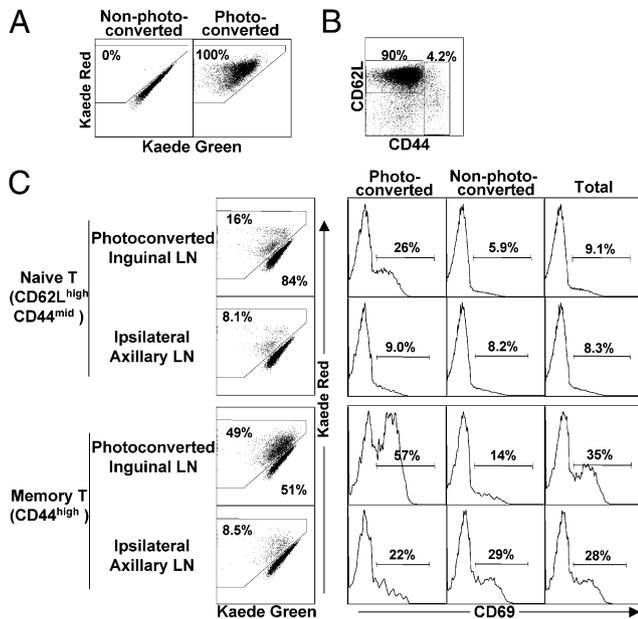
FIGURE 3. Analysis of CD69 expression and migration of naive and memory CD4^{+} T cells in Kaede-Tg mice. *A*, Photoconversion of cells in inguinal LN. Inguinal LN was exposed (*right panel*) to violet light or was not exposed (*left panel*) and analyzed for the photoconversion of Kaede, as described in *Materials and Methods*. *B*, Cells from PLNs were stained with anti-CD4, anti-CD25, anti-CD62L, and anti-CD44 mAbs and analyzed by flow cytometry. The expression of CD44 and CD62L in CD4^{+}CD25^{−} gated cells is shown. *C*, CD69 expression of naive and memory CD4^{+} T cells in photoconverted inguinal LN and connecting ipsilateral axillary LN. Inguinal LN was exposed to violet light as described in *Materials and Methods*. Twenty-four hours after the photoconversion, cells from photoconverted inguinal LN and connecting ipsilateral axillary LN were stained with anti-CD4, anti-CD25, anti-CD62L, anti-CD44, and anti-CD69 mAbs. Cells were separated into naive and memory CD4^{+} T cells on the basis of the surface expression of CD62L and CD44, as shown in *B*, and CD69 expression was analyzed in photoconverted and nonphotoconverted T cell populations. Three animals were independently analyzed for each group, with similar results, and the results of one representative analysis are shown.

are the remaining cells in the inguinal LNs from the time of photoconversion. In the axillary LNs that are connected directly to the inguinal LNs via the lymphatic system (20), photoconverted cells are recent migrants from the inguinal LNs. In this analysis, CD4^{+} T cells were also separated into naive and memory T cells based on their surface expression of CD62L and CD44 molecules (Fig. 3*B*). As shown in Fig. 3*C*, naive CD4^{+} T cells in the inguinal LNs were rapidly replaced; 24 h after the photoconversion, only 16% of the cells remained in the inguinal LNs. At this time, the cells that emigrated from the inguinal LNs could be found in the axillary LNs (8.1%). The percentage of cells expressing CD69 in the remaining population (photoconverted cells) was significantly greater than that of the recently immigrated naive CD4^{+} T cell population (nonphotoconverted cells; 26% versus 5.9%). Naive CD4^{+} T cells that migrated to the axillary LNs contained a percentage of CD69^{+} cells that was similar to the naive T cell population present in the LNs. These results suggest that CD69 expression promotes T cell retention in LNs by downregulating the migratory response to S1P1 in the steady state. To show that the modulation of CD69 expression is functionally significant for the migration of naive CD4^{+} T cells, we evaluated the migration activity of CD69^{−} and CD69^{+} CD4^{+} T cells to sphingosine-1-phosphate in a Transwell assay. As shown in Supplemental Fig. 4, CD69^{−} and CD69^{+} CD4^{+} T cells migrated to CCL19 at a comparable level. However, CD69^{−}, but not CD69^{+}, CD4^{+} T cells

migrated to sphingosine-1-phosphate. These results indicate that CD69 expression is of functional significance for the migration of naive CD4^{+} T cells in the nonimmunized condition.

In the case of memory CD4^{+} T cells, replacement in the initial 24 h was significantly slower than that of naive CD4^{+} T cells (84% for naive T cells and 51% for memory T cells) (Fig. 3*C*). Again, the remaining cells contained a significantly greater percentage (57%) of CD69^{+} cells than did the cells that immigrated to the LNs (14%). In axillary LNs, photoconverted memory CD4^{+} T cells from the inguinal LN and nonphotoconverted CD4^{+} T cells had similar percentages of CD69^{+} cells (22% versus 29%). These results demonstrate that naive and memory CD4^{+} T cell subsets have distinct migratory kinetics, and the expression of CD69 in each population is regulated differently.

The above kinetic analysis revealed a clear relationship between migration/emigration of CD4^{+} T cells and the expression of CD69. As shown in Fig. 4*A*, naive CD4^{+} T cells emigrated from the inguinal LN much faster than did memory T cells. This difference in emigration from the photoconverted LNs coincided with the migration of CD4^{+} T cells to the connecting ipsilateral axillary LNs. The migration of naive CD4^{+} T cells to ipsilateral axillary LNs peaked at 6 h after photoconversion, whereas the migration of memory T cells was delayed, reaching its peak at 12 h after photoconversion. The percentage of CD69-expressing cells gradually increased in the remaining CD4^{+} T cell population in the inguinal LN (Fig. 4*B*, *left panel*). This finding, which is in agreement with those of previous studies (16–18), indicates that CD69 plays a critical role in the retention of T cells in the LN, and cells expressing this molecule preferentially remain in the photoconverted inguinal LNs. At the same time, the percentage of
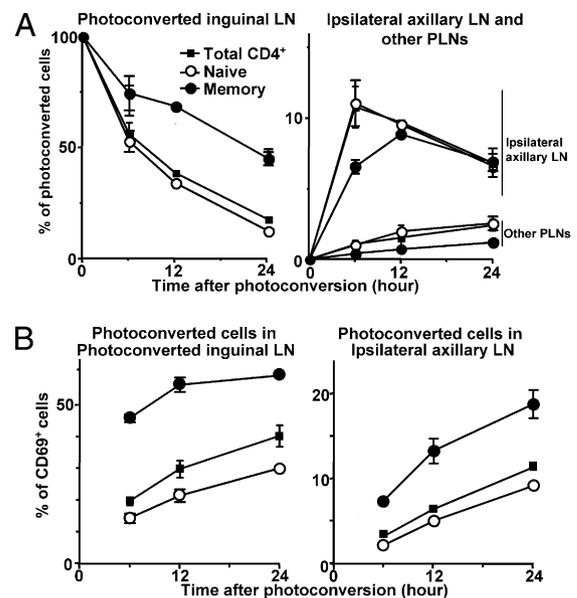

FIGURE 4. Time course of naive and memory CD4^{+} T cell migration and CD69 expression. *A* and *B*, Inguinal LNs were photoconverted as in Fig. 3*C*. At indicated times after the photoconversion, cells from photoconverted inguinal LN, ipsilateral axillary LN, and the pool of other nonphotoconverted PLNs (contralateral popliteal, inguinal, bilateral axillary, brachial, and cervical LNs) were analyzed for the expression of surface markers and the presence of photoconverted Kaede in different populations, using the method described in Fig. 3*C*. More than three animals were independently analyzed, and the percentage of photoconverted cells (mean ± SE) in each population (*A*) and the percentage of CD69^{+} cells in the photoconverted cells (*B*) in the photoconverted inguinal LN (*left panel*) and the ipsilateral axillary LNs (*right panel*) are shown as mean ± SE.

CD69-expressing cells also increased in the photoconverted CD4^{+} T cells in the ipsilateral axillary LN, which had migrated from the inguinal LN (Fig. 4B, right panel).

In situ upregulation of CD69 in T cells migrating to LN

The increase in the number of CD69^{+} cells in the population that migrated to the axillary LN suggests that CD69 is induced by the in situ interaction between T cells and endogenous Ags. However, it is possible that cells expressing CD69 migrate to the same LN with delayed kinetics and accumulate in a time-dependent manner. To distinguish between these two possibilities, we treated mice with the S1P1 agonist, FTY720 (18, 26), which inhibits the emigration of CD4^{+} T cells from LNs 6 h after inguinal LN photoconversion, and we analyzed cell migration and upregulation of CD69 molecules 24 h after photoconversion (Fig. 5). Six hours after photoconversion, 42% of naive CD4^{+} T cells in the inguinal LNs were replaced, and, at the same time, 16% of naive CD4^{+} T cells in the axillary LNs were from the photoconverted inguinal LNs. Among the cells that migrated to the axillary LNs, 2.2% expressed CD69. In parallel experiments, inguinal LNs were photoconverted, and mice were treated with FTY720 6 h after photoconversion. Cell migration and CD69 expression were analyzed 24 h after photoconversion. The percentage of photoconverted naive CD4^{+} T cells in the inguinal LNs (58%) was similar to that of cells analyzed 6 h after photoconversion but without FTY720 treatment (58%). There also was no increase in the percentage of photoconverted naive CD4^{+} T cells in the axillary LNs treated with FTY720 (16% versus 16%). These results indicate that cell migration is efficiently inhibited by treatment with FTY720 after the initial migration in the first 6 h. When cell migration was inhibited 6 h after photoconversion, the percentage of photoconverted naive CD4^{+} T cells expressing CD69 in the axillary LNs increased from 2.2% to 6.1%. These findings strongly indicate that cells migrating to the axillary LNs do not express CD69, although the expression is induced in the new LN environment by interaction with endogenous Ags.

In the case of memory CD4^{+} T cells, only 19% of the cells were replaced in the inguinal LNs in the initial 6 h after photoconversion. FTY720 treatment also inhibited the additional migration of memory CD4^{+} T cells, similar to naive T cells. CD69 expression on memory CD4^{+} T cells increased from 7.4% at 6 h to 19% at 24 h after photoconversion, with no apparent cell migration between 6 and 24 h. Thus, memory CD4^{+} T cells also increase CD69^{+} populations in situ in the LN.
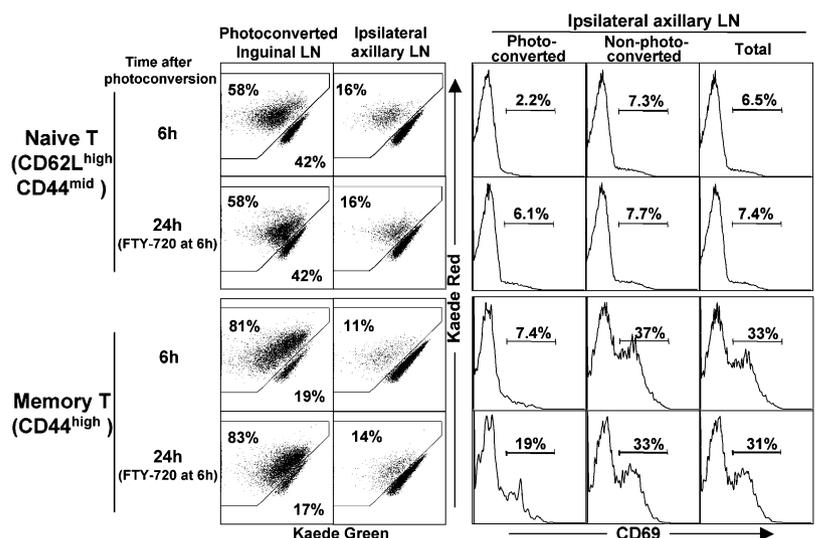
Induction of CD69 expression on CD4^{+} T cells and retention of CD4^{+} T cells in LN are sustained by interaction with endogenous Ags

We have suggested that CD69 expression on CD4^{+} T cells is induced by interaction with endogenous Ags, and cells that interact with endogenous Ags are retained in lymphoid organs. We tried to confirm the role of endogenous Ags in T cell sequestration into lymphoid organs. Endogenous Ag presentation to CD4^{+} T cells was thought to be generated by the MHC class II + self-peptide complex. CD4^{+} T cells were purified from Kaede-Tg mice and transferred to WT and MHC class II KO mice. Four days later, inguinal LNs were photoconverted; 12 h later, CD69 expression levels and the remaining transferred CD4^{+} T cells in the photoconverted LNs were analyzed. As shown in Fig. 6, 19% of transferred CD4^{+} T cells in WT mice expressed CD69. However, CD69 expression on the transferred CD4^{+} T cells was almost completely downregulated when the cells were transferred to MHC class II KO mice. Consistent with CD69 expression, 40% of the transferred CD4^{+} T cells remained in the WT host. However, only 14% of the cells remained in the photoconverted LNs in the MHC class II KO host, indicating that CD4^{+} T cells leave LNs more rapidly in the absence of TCR-self-peptide–MHC interactions. Thus, these results indicated that endogenous Ags play a crucial role in CD69 expression and the sequestration of CD4^{+} T cells into lymphoid organs in the steady state.

Functional difference between CD69^{+} and CD69^{−} CD4^{+} T cells

It was shown that T cells undergoing homeostatic expansion in lymphopenic mice change their surface phenotype (27–29) and acquire memory-like function (28–30). Thus, we tested whether there is a functional difference between CD69^{+} and CD69^{−} naive CD4^{+} T cells (Fig. 7). Naive CD4^{+} T cells from normal mice were sorted into CD69^{+} and CD69^{−} populations, and 5×10^4 cells of each population were stimulated with plate-bound anti-CD3 mAb. CD69^{+} CD4^{+} T cells exhibited rapid production of IL-2 and TNF-α, and the magnitude of cytokine production by the CD69^{+} population, especially IL-2, was significantly larger than that by CD69^{−} T cells. CD69^{−} T cells were capable of producing significant amounts of cytokines when a large number of cells (5×10^5 cells/well) were used for the assay. Thus, the use of a very small number of T cells for the assay revealed a kinetic difference in cytokine production between CD69^{+} and CD69^{−} populations. We cannot exclude completely the possibility that the biotinylated anti-CD69 mAb cross-linked by streptavidin transmits some

FIGURE 5. In situ expression of CD69 on CD4^{+} T cells in LN. Inguinal LNs were photoconverted as in Fig. 3C. Six hours after the photoconversion, cells from photoconverted inguinal and ipsilateral axillary LNs were analyzed for the expression of CD69, according to the method described in Fig. 3C. Some mice received FTY720 (1 mg/kg, i.p.) 6 h after the photoconversion, and cells were analyzed for CD69 expression 24 h after the photoconversion. Three animals were independently analyzed for each group, with similar results, and the results of one representative analysis are shown.
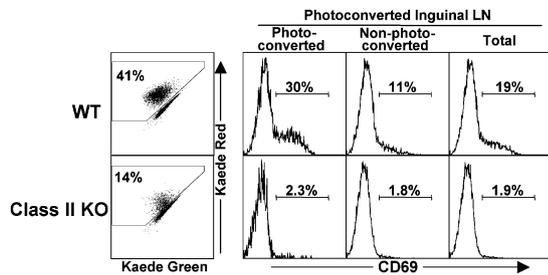
FIGURE 6. Induction of CD69 expression on CD4^{+} T cells and retention of CD4^{+} T cells in LN are sustained by class II molecules. CD4^{+} T cells were purified from Kaede-Tg mice and transferred to WT and MHC class II KO mice. Four days later, inguinal LNs were photoconverted, and 12 h later, CD69 expression and the photoconverted population of transferred CD4^{+} T cells in the photoconverted LNs were analyzed. Percentages of photoconverted cells in the transferred CD4^{+} T cells and CD69 expression of photoconverted, nonphotoconverted, and total CD4^{+} T cell populations are shown. Three animals were independently analyzed for each group, with similar results, and the results of one representative analysis are shown.

signals via the CD69 molecule. However, a previous study using highly purified T cells clearly indicated that the enhancement of plate-bound anti-CD3 T cell activation by anti-CD69 Ab requires cross-linking of anti-CD69 and other APC-derived signals (11). We stimulated sorted cells with plate-bound anti-CD3 Ab alone. Thus, we think that the high response of CD69^{+} cells compared with CD69^{−} cells after TCR stimulation is an intrinsic characteristic and is not caused by signal transduction via CD69.

Discussion

The interaction with endogenous Ags in the thymus plays a critical role in the selection of T lymphocytes. T cells that interact with endogenous Ags with high affinity are negatively selected. Immature thymocytes that interact with the endogenous Ag/MHC complex with certain affinity are selected to mature into CD4 or CD8 single-positive T cells that can recognize foreign Ag peptides presented by self-MHC. This selection using the endogenous Ag/MHC complex is essential to generate a T cell repertoire that can mount an immune response to foreign Ags without causing an autoimmune T cell response to endogenous Ags (31, 32).

The interaction between mature T cells and the endogenous Ag/MHC complex was initially demonstrated in transfer experiments using lymphopenic recipients. CD4^{+} and CD8^{+} T cells proliferated in vivo, when transferred to mice lacking T lymphocytes in class II and class I MHC-dependent manners, respectively (27, 33–37). Furthermore, the survival of T cells in vivo was shown to be de-

pendent on the presence of MHC, and the reactivity of naive CD4^{+} T cells to foreign Ags in vitro was influenced by the interaction between T cells and class II MHC in vivo (28, 29, 38–40). At present, neither the nature of endogenous Ags interacting with T cells nor the precise biochemical events induced by this interaction is well characterized (28, 29). However, all of the results suggested that the function and maintenance of naive T cells in the periphery require the interaction between T cells and the endogenous Ag/MHC complex. We have demonstrated that DO11.10 T cells that recognize OVA-derived peptide in the context of I-A[d] class II MHC interact with endogenous Ags in vivo and express the early activation marker CD69. The percentage of CD69^{+} DO11.10 T cells in vivo is inversely related to the number of cells transferred (Fig. 1B), and the expression of CD69 on DO11.10 T cells can compete by cells expressing the same TCR but not by T cells with diverse TCR expression (Fig. 1C). The same phenomenon was found in T cells expressing Tg TCR with different Ag specificities (Supplemental Fig. 2). The number of CD69^{+} DO11.10 T cells is relatively constant, regardless of the total number of DO11.10 T cells (Supplemental Table I). Thus, these results strongly indicate that T cells compete for limited endogenous Ag/MHC complexes in lymphoid organs for interaction via clonally distributed αβ TCR. The nature of endogenous Ags that interact with DO11.10 TCR is difficult to characterize. However, there is no difference in the induction of CD69 in DO11.10 T cells between germ-free recipients and recipients under normal SPF conditions (Supplemental Fig. 5), ruling out the involvement of Ags from gut bacterial flora. It is possible that positive selection in the thymus and activation of mature T cells in the periphery might share the Ags, because transient CD69 expression was observed in the positively selected thymocytes (14) and in T cells interacting with endogenous Ags in the peripheral lymphoid organs. The identification of endogenous Ags that interact with T cells expressing well-characterized TCR would provide new insights into the relationship between thymic selection and peripheral activation of T cells. Further experiments using new approaches are required.

In normal mice, CD4^{+} T cell populations contain a significant number of CD69^{+} cells in the nonimmunized condition (Fig. 2A). Because the same percentage of CD69^{+} CD4^{+} T cells was found in the peripheral lymphoid organs (except in Peyer's patches) of germ-free mice, the effect of gut bacteria seems to be negligible. However, CD69 expression on CD4^{+} T cells is not a stable phenotype. As shown in the transfer experiments, a fraction of the CD69^{−} population became positive 4 d after transfer into normal recipients. In contrast, the CD69^{+} population readily lost the expression under the same transfer conditions (Fig. 2C). These
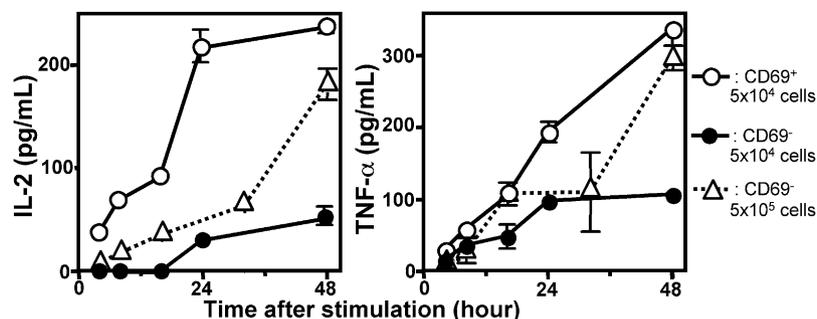
FIGURE 7. Functional difference between CD69^{+} and CD69^{−} naive CD4^{+} T cells. Negatively selected CD4^{+} T cells were stained with biotinylated anti-CD69 mAb, followed by fluorochrome-conjugated streptavidin, anti-CD4, anti-CD25, anti-CD44, and anti-CD62L. Stained cells were sorted into CD69^{−} and CD69^{+} CD25^{−}CD62L^{high}CD44[midlow] naive CD4^{+} T cell populations with a fluorescence-activated cell sorter, as described in *Materials and Methods*. Indicated numbers of sorted cells were stimulated with plate-coated anti-CD3 mAb. At indicated hours of in vitro culture, the supernatants were harvested, and IL-2 and TNF-α concentrations in the supernatants were measured as described in *Materials and Methods*.

results suggest that conventional CD4+ T cells, similar to DO11.10 T cells, also compete for interaction with endogenous Ags to induce the expression of CD69. This notion is further supported by the fact that no CD69 upregulation on normal CD4+ T cells (Fig. 5) or TCR-Tg T cells (30) was observed in cells that were transferred to MHC class II− recipients.

The use of Kaede-Tg mice revealed the relationship between cell migration and interaction with endogenous Ags without cell transfer. First, all circulating T cells in blood and lymph had the CD69− phenotype (Fig. 2A), and a combination of photo-conversion of Kaede and the use of FTY720 demonstrated that the number of CD69-expressing cells increased in the new immigrant CD4+ T cell population, without a new influx of photoconverted cells in the LN (Fig. 5). Analysis of the emigration of CD4+ T cells from LNs also demonstrated that CD69-expressing cells remained in the LN much longer than did the CD69− population (Fig. 3). From these results, one can envision that lymphocytes migrate through the whole body in search of endogenous Ags to interact with in the lymphoid organs. In each lymphoid organ, T cells compete with each other for the limited endogenous Ags. Successful interaction with endogenous Ags induces the expression of CD69 molecules on T cells for prolonged retention in the lymphoid organs. However, CD69 expression is transient, and the loss of CD69 expression leads to emigration of the T cells. T cells that fail to interact with endogenous Ags in the LN emigrate rapidly through lymph or bloodstream to other lymphoid organs, where they continue their search for endogenous Ags.

The interaction with endogenous Ags induces CD69 expression for prolonged retention in the LN and changes the function of naive CD4+ T cells. It was demonstrated that the expansion of naive CD4+ T cells in the lymphopenic condition coincides with changes in their surface phenotype (27–29) and in response to stimulation through TCR (27–30). Our results indicate that even under physiological conditions, naive CD4+ T cells interact with endogenous Ags to change their phenotype and functions. The phenomenon observed in the lymphopenic condition may simply be an exaggeration of the continuous interaction between naive CD4+ T cells and endogenous Ags demonstrated in this study. It is difficult to evaluate the importance of the change in function of naive T cells in response to pathogens, although the increased responsiveness of naive CD4+ T cells induced by endogenous Ags may play an important role in preparing T cell response to foreign Ags, as suggested by a previous study with TCR-Tg mice (40). Further experiments with a pathogen-specific system are required to address this issue.

In the experiments using Kaede-Tg mice, naive and memory phenotype T cells had similar migration and emigration patterns based on CD69 expression. In both populations, only cells lacking CD69 emigrate from the lymphoid organs to other lymphoid organs, and some, but not all, T cells interact with endogenous Ags to express CD69 in situ. However, the percentage of cells expressing CD69 is much larger in the memory phenotype T cells than in the naive T cell population. This is reflected by the much slower emigration of memory-type T cells from the lymphoid organs compared with naive T cells. This difference may be a reflection of the difference in the threshold for activation by endogenous Ags between memory and naive T cells. We do not know the Ags responsible for the generation of memory phenotype T cells in nonimmunized mice under SPF and germ-free conditions. Nevertheless, it is possible that the continuous interaction with endogenous Ags plays an important role in the survival of memory T cells and the maintenance of immunological memory in the absence of original pathogens.

In summary, T cells move through lymphoid organs, interact with endogenous Ags, and change their function to maintain better reactivity to foreign Ags in the steady state. Our results are the first to demonstrate that the steady-state migration of T cells is not a passive movement prior to encountering foreign Ags; rather, T cells recirculate to search endogenous Ags in a limited niche. The role of this T cell–endogenous Ag interaction under pathogenic conditions, such as infection with pathogens and the initiation of autoimmune response, awaits further investigation.

Disclosures

References

1. Gowans, J. L. 1959. The recirculation of lymphocytes from blood to lymph in the rat. *J. Physiol.* 146: 54–69.
2. Cahill, R. N., D. C. Poskitt, D. C. Frost, and Z. Trnka. 1977. Two distinct pools of recirculating T lymphocytes: migratory characteristics of nodal and intestinal T lymphocytes. *J. Exp. Med.* 145: 420–428.
3. Mackay, C. R., W. G. Kimpton, M. R. Brandon, and R. N. Cahill. 1988. Lymphocyte subsets show marked differences in their distribution between blood and the afferent and efferent lymph of peripheral lymph nodes. *J. Exp. Med.* 167: 1755–1765.
4. Stekel, D. J., C. E. Parker, and M. A. Nowak. 1997. A model of lymphocyte recirculation. *Immunol. Today* 18: 216–221.
5. Mora, J. R., and U. H. von Andrian. 2006. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol.* 27: 235–243.
6. Kabelitz, D., and R. Medzhitov. 2007. Innate immunity—cross-talk with adaptive immunity through pattern recognition receptors and cytokines. *Curr. Opin. Immunol.* 19: 1–3.
7. Fearon, D. T., and R. M. Locksley. 1996. The instructive role of innate immunity in the acquired immune response. *Science* 272: 50–53.
8. von Andrian, U. H., and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Natl. Rev. Immunol.* 3: 867–878.
9. Lee, H. K., and A. Iwasaki. 2007. Innate control of adaptive immunity: dendritic cells and beyond. *Semin. Immunol.* 19: 48–55.
10. Christensen, J. E., and A. R. Thomsen. 2009. Co-ordinating innate and adaptive immunity to viral infection: mobility is the key. *APMIS* 117: 338–355.
11. Yokoyama, W. M., F. Koning, P. J. Kehn, G. M. Pereira, G. Stingl, J. E. Coligan, and E. M. Shevach. 1988. Characterization of a cell surface-expressed disulfide-linked dimer involved in murine T cell activation. *J. Immunol.* 141: 369–376.
12. Testi, R., J. H. Phillips, and L. L. Lanier. 1989. Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. Requirement for receptor cross-linking, prolonged elevation of intracellular [Ca++] and stimulation of protein kinase C. *J. Immunol.* 142: 1854–1860.
13. Ziegler, S. F., F. Ramsdell, and M. R. Alderson. 1994. The activation antigen CD69. *Stem Cells* 12: 456–465.
14. Wang, C. R., K. Hashimoto, S. Kubo, T. Yokochi, M. Kubo, M. Suzuki, K. Suzuki, T. Tada, and T. Nakayama. 1995. T cell receptor-mediated signaling events in CD4+CD8+ thymocytes undergoing thymic selection: requirement of calcineurin activation for thymic positive selection but not negative selection. *J. Exp. Med.* 181: 927–941.
15. Nakayama, T., D. J. Kasprowicz, M. Yamashita, L. A. Schubert, G. Gillard, M. Kimura, A. Didierlaurent, H. Koseki, and S. F. Ziegler. 2002. The generation of mature, single-positive thymocytes in vivo is dysregulated by CD69 blockade or overexpression. *J. Immunol.* 168: 87–94.
16. Shiow, L. R., D. B. Rosen, N. Brdicková, Y. Xu, J. An, L. L. Lanier, J. G. Cyster, and M. Matloubian. 2006. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 440: 540–544.
17. Kabashima, K., N. M. Haynes, Y. Xu, S. L. Nutt, M. L. Allende, R. L. Proia, and J. G. Cyster. 2006. Plasma cell S1P1 expression determines secondary lymphoid organ retention versus bone marrow tropism. *J. Exp. Med.* 203: 2683–2690.
18. Cyster, J. G. 2005. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu. Rev. Immunol.* 23: 127–159.
19. Pham, T. H., T. Okada, M. Matloubian, C. G. Lo, and J. G. Cyster. 2008. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. *Immunity* 28: 122–133.
20. Tomura, M., N. Yoshida, J. Tanaka, S. Karasawa, Y. Miwa, A. Miyawaki, and O. Kanagawa. 2008. Monitoring cellular movement in vivo with photo-convertible fluorescence protein "Kaede" transgenic mice. *Proc. Natl. Acad. Sci. USA* 105: 10871–10876.
21. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* 250: 1720–1723.
22. Berg, L. J., A. M. Pullen, B. Fazekas de St Groth, D. Mathis, C. Benoist, and M. M. Davis. 1989. Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell* 58: 1035–1046.
23. Takase, M., E. M. Kanagawa, and O. Kanagawa. 2007. Age-dependent TCR revision mediated by interaction between alphabeta TCR and self-antigens. *J. Immunol.* 179: 2163–2169.
24. Madsen, L., N. Labrecque, J. Engberg, A. Dierich, A. Svejgaard, C. Benoist, D. Mathis, and L. Fugger. 1999. Mice lacking all conventional MHC class II genes. *Proc. Natl. Acad. Sci. USA* 96: 10338–10343.

25. Hataye, J., J. J. Moon, A. Khoruts, C. Reilly, and M. K. Jenkins. 2006. Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science* 312: 114–116.

26. Chiba, K., Y. Yanagawa, Y. Masubuchi, H. Kataoka, T. Kawaguchi, M. Ohtsuki, and Y. Hoshino. 1998. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J. Immunol.* 160: 5037–5044.

27. Ernst, B., D. S. Lee, J. M. Chang, J. Sprent, and C. D. Surh. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* 11: 173–181.

28. Jameson, S. C. 2002. Maintaining the norm: T-cell homeostasis. *Natl. Rev. Immunol.* 2: 547–556.

29. Surh, C. D., and J. Sprent. 2005. Regulation of mature T cell homeostasis. *Semin. Immunol.* 17: 183–191.

30. Tanchot, C., A. Le Campion, S. Léaument, N. Dautigny, and B. Lucas. 2001. Naive CD4(+) lymphocytes convert to anergic or memory-like cells in T cell-deprived recipients. *Eur. J. Immunol.* 31: 2256–2265.

31. von Boehmer, H., I. Aifantis, F. Gounari, O. Azogui, L. Haughn, I. Apostolou, E. Jaeckel, F. Grassi, and L. Klein. 2003. Thymic selection revisited: how essential is it? *Immunol. Rev.* 191: 62–78.

32. Davis, M. M., M. Krogsgaard, M. Huse, J. Huppa, B. F. Lillemeier, and Q. J. Li. 2007. T cells as a self-referential, sensory organ. *Annu. Rev. Immunol.* 25: 681–695.

33. Viret, C., F. S. Wong, and C. A. Janeway, Jr. 1999. Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. *Immunity* 10: 559–568.

34. Bender, J., T. Mitchell, J. Kappler, and P. Marrack. 1999. CD4+ T cell division in irradiated mice requires peptides distinct from those responsible for thymic selection. *J. Exp. Med.* 190: 367–374.

35. Oehen, S., and K. Brduscha-Riem. 1999. Naïve cytotoxic T lymphocytes spontaneously acquire effector function in lymphocytopenic recipients: A pitfall for T cell memory studies? *Eur. J. Immunol.* 29: 608–614.

36. Goldrath, A. W., and M. J. Bevan. 1999. Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity* 11: 183–190.

37. Kieper, W. C., and S. C. Jameson. 1999. Homeostatic expansion and phenotypic conversion of naïve T cells in response to self peptide/MHC ligands. *Proc. Natl. Acad. Sci. USA* 96: 13306–13311.

38. Polic, B., D. Kunkel, A. Scheffold, and K. Rajewsky. 2001. How alpha beta T cells deal with induced TCR alpha ablation. *Proc. Natl. Acad. Sci. USA* 98: 8744–8749.

39. Labrecque, N., L. S. Whitfield, R. Obst, C. Waltzinger, C. Benoist, and D. Mathis. 2001. How much TCR does a T cell need? *Immunity* 15: 71–82.

40. Stefanová, I., J. R. Dorfman, and R. N. Germain. 2002. Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. *Nature* 420: 429–434.