Early Development in the Peritoneal Cavity of CD49d<sup>high</sup> Th1 Memory Phenotype CD4<sup>+</sup> T Cells with Enhanced B Cell Helper Activity

Hana Moon, Chanho Park, Jae-Ghi Lee, Sang Hyuck Shin, Joo Hee Lee, Inseong Kho, Kyeong Jin Kang, Hoon-Suk Cha and Tae Jin Kim

*J Immunol* published online 8 June 2015
http://www.jimmunol.org/content/early/2015/06/05/jimmunol.1401661

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/06/05/jimmunol.1401661.DCSupplemental

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
Early Development in the Peritoneal Cavity of CD49d<sub>high</sub> Th1 Memory Phenotype CD4<sup>+</sup> T Cells with Enhanced B Cell Helper Activity

Hana Moon,* Chanho Park,* Jae-Ghi Lee,* Sang Hyuck Shin,* Joo Hee Lee,* Inseong Kho,* KyeongJin Kang,† Hoon-Suk Cha,‡ and Tae Jin Kim*

The Th cells that regulate peritoneal B-1 cell functions have not yet been well characterized. To address this question, we investigated peritoneal CD4<sup>+</sup> T cells, observed a high frequency of the conjugates of B-CD4<sup>+</sup> T cells in the peritoneal cavity, and identified a population of CD49d<sub>high</sub>CD4<sup>+</sup> T cells that constituted about half of all CD4<sup>+</sup> T cells in the peritoneal cavity, but were rarely found in other compartments. Peritoneal CD49d<sub>high</sub>CD4<sup>+</sup> T cells were CD44<sub>high</sub>CD62L<sub>low</sub>; expressed integrin α<sub>4</sub>β<sub>1</sub> and CXCR3; and rapidly secreted IFN-γ, TNF-α, and IL-2, showing features of proinflammatory Th1 cells. Peritoneal CD49d<sub>high</sub>CD4<sup>+</sup> T cells developed spontaneously, were detected at the age of 12 d, and showed stem cell–like properties. Their development was observed in mice deficient for signaling lymphocytic activation molecule-associated protein, but not in athymic nude mice and mice lacking in expression of MHC class II on thymic epithelial cells. Peritoneal CD49d<sub>high</sub>CD4<sup>+</sup> T cells were more resistant to irradiation and more sensitive to NAD-induced cell death than CD49d<sub>low</sub>CD4<sup>+</sup> T cells. Notably, peritoneal CD49d<sub>high</sub>CD4<sup>+</sup> T cells also showed some characteristics of follicular Th cells, such as the expression of programmed cell death 1, ICOS, IL-21, and CXCR5. Moreover, peritoneal CD49d<sub>high</sub>CD4<sup>+</sup> T cells enhanced the secretion of IgM Abs by B-1a cells and IgG Abs by splenic B cells. These data suggest that peritoneal CD49d<sub>high</sub>CD4<sup>+</sup> T cells may be innate-like CD4<sup>+</sup> T cells, which develop early and have a dual capacity to support both humoral and cellular immunity. *The Journal of Immunology, 2015, 195: 000–000.

The peritoneal cavity is a unique compartment from which infections can easily spread to adjacent organs, such as the intestine and liver. The peritoneal cavity readily communicates with the systemic blood circulation through the omental milky spot, which is a lymphoid tissue embedded in omental fat (1). The peritoneal cavity harbors many immune cells, including macrophages, mast cells, T cells, and B cells. Moreover, the peritoneal cavity is a resource-rich niche for the survival of B-1 cells, which constitutively secrete natural Abs (2). Whereas peritoneal B-2 cells have been reported to have B-1 cell–like characteristics compared with splenic lymph node B-2 cells (3), little attention has been given to peritoneal T cells. Furthermore, there are only a few reports that describe CD4<sup>+</sup> T cells providing help to B-1 cells (4).

Beyond the conventional helper functions for B cells and CD8<sup>+</sup> T cells, memory CD4<sup>+</sup> T cells exert potent inflammatory actions; furthermore, these actions are independent from their TCR specificities (5, 6). In neonates, innate CD4<sup>+</sup> T cells are believed to substitute for this function of memory CD4<sup>+</sup> T cells. Innate T cells are usually defined as memory phenotype (MP) T cells that are generated within the thymus, such as invariant NKT (iNKT) cells (7). Although memory CD4<sup>+</sup> T cells are traditionally divided into central memory CD62L<sub>high</sub>CCR7<sup>+</sup>CD4<sup>+</sup> T cells and effector memory CD62L<sub>low</sub>CCR7<sup>−</sup>CD4<sup>+</sup> T cells (8), memory CD4<sup>+</sup> T cells were recently shown to be more heterogeneous with respect to their migratory and functional properties than was previously thought (9, 10). Notably, distinct memory CD4<sup>+</sup> T cell populations such as tissue-resident memory and bone marrow–resident memory CD4<sup>+</sup> T cells have been described, which suggests complicated pathways of memory T cell development and maintenance (11, 12).

Some MP T cells have been observed to develop spontaneously and do not appear to differentiate from naive T cells upon antigenic stimuli. Although MP T cells are broadly categorized as innate T cells, the precise pathways driving the development of MP T cells have not yet been elucidated. The spontaneous generation of a distinct subpopulation of MP CD44<sub>high</sub>CD122<sup>−</sup>CD8<sup>+</sup> T cells, in the absence of specific priming events and in a manner distinct from the generation of bona fide Ag-experienced CD44<sub>high</sub>CD122<sup>−</sup> memory CD8<sup>+</sup> T cells, has been reported (13, 14). Several reports have also noted the presence of nonconventional MP CD4<sup>+</sup> T cells; however, the functional significance of these cells is still unclear (15–17). In this study, we report the spontaneous generation of MHC class II–dependent MP CD4<sup>+</sup> T cells. These MP CD4<sup>+</sup> T cells accumulated rapidly in the peritoneal cavity during early development, maintained a persistent population even after neonatal thymectomy, and were able to enhance the Ab secretion by B-1a cells.

---

*Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Gyeonggi-do 440-746, Republic of Korea; †Department of Anatomy and Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Gyeonggi-do 440-746, Republic of Korea; and ‡Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Republic of Korea

Received for publication June 30, 2014. Accepted for publication May 15, 2015.

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF-2011-0009365), which was funded by the Ministry of Education, Science, and Technology, Republic of Korea, and by a Samsung Biomedical Research Institute Grant.

Address correspondence and reprint requests to Prof. Tae Jin Kim, Department of Molecular Cell Biology, Division of Immunobiology, Sungkyunkwan University School of Medicine, Suwon, Gyeonggi-do 440-746, Republic of Korea. E-mail address: tjkim@skku.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: Bcl-6, B cell lymphoma 6; FSC, forward scatter; iNKT, invariant NKT; MP, memory phenotype; qRT-PCR, quantitative RT-PCR; SAP, SLAM-associated protein; SLAM, signaling lymphocyte activating molecule; Thy, follicular Th; WT, wild-type.

Copyright © 2015 by The American Association of Immunologists, Inc.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1401661
Materials and Methods

**Mice, irradiation, and neonatal thymectomy**

C57BL/6 wild-type (WT) and BALB/c nude mice were purchased from Orient Bio (Sungnam, Korea). Cd49dlow mice, Sh2d2alow mice, and mice carrying a deletion in promoter IV of the Mhc2ta gene for CITA (CITAPlv+/-) were provided by L. Van Kaer (Vanderbilt University School of Medicine, Nashville, TN), C. Terhorst (Beth Israel Deaconess Medical Center, Boston, MA), and H. Acha-Orbea (University of Lausanne, Switzerland), respectively. Cd49dlow mice were purchased from The Jackson Laboratory (Bar Harbor, ME). This study was approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University School of Medicine. All procedures were performed in a pathogen-free facility, according to institutional guidelines.

**Three-day-old Cd49dlow mice** were thymectomized by the suction technique. At 4 wk of age, peritoneal cells were collected. For total body γirradiation, Cd49d mice were irradiated with a 105 Cs Gamacell source (IBL 437C; CIS Bio International, Bangnols sur Ceze, France) until a dose of 12 Gy was reached.

**Antibodies**

Abs against CD3 (90, NK.1.1 (PK136), IL-2 (JES6-5H4), PLFV (R17-809), Vβ2 (B2.206), Vβ3 (KJ25), Vβ4 (KT4), Vβ3.1 and 5.2 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR3.10), Vβ8.1 and 8.2 (MR5-2), Vβ3 (1B3.3), Vβ9 (MR10-2), Vβ10 (B21.5), Vβ11 (RR3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), and Vβ14 (19-2) were purchased from BD Biosciences (San Jose, CA); Abs against CD44 (M7), CD202 (MEL-14), CD150 (signaling lymphocyte activator molecule [SLAM], TC15-12F12), ICOS (7E.17G9), CD122 (SH4), CCRX3 (C3CR3-173), CD127 (A7R34), IL-4 (11B11), TGF-β (TW7-2089), CD27 (LG3.1A10), CD19 (6D5), CD11b (M1/70), and CD5 (53-73) were from BioLegend (San Diego, CA); and Abs against CD4 (RM4.5), CD49d (9C10), β1 integrin (HMB-1), αβ integrin (DATK32), CCR7 (4B12), programmed cell death 1 (29F.1A12), TCR-β (H57-597), IFN-γ (XM1G.1), IL-5 (TRFK5), IL-10 (JES5-16E3), IL-13 (eBio13A), and TNF-α (M6P-XT22) were from eBioscience (San Diego, CA).

**Flow cytometric analysis and cell sorting**

Peritoneal cells were isolated by flushing the peritoneal cavity with PBS. Single-cell suspensions were prepared from the spleen, bone marrow, thymus, inguinal lymph nodes, and blood through mechanical disruption and passage of the homogenate through a nylon membrane. Cells were stained on ice for 15 min with the appropriate combinations of fluorochrome-conjugated Abs in FACS buffer (5% BCS and 0.05% sodium azide in PBS). After washing with FACS buffer, the stained cells were analyzed on a FACS Canto II system (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, San Carlos, CA). In all cases, doublets (based on forward scatter [FSC]-area versus FSC-width) were excluded, except as indicated in Fig. 1.

**In vitro transfer of effector cells**

For in vitro transfer experiments, cells were sorted using a FACSaria III (BD Biosciences) system. Peritoneal Cd49dlo‐Cd49d mice or Cd49dw‐Cd49d+ T cells, or splenic Cd49dlo‐Cd49d+ T cells were sorted after staining with anti‐CD4 and anti‐CD49d Abs. Peritoneal B1a cells were sorted after staining with anti‐CD19, anti‐CD5, and anti‐CD11b Abs. The staining with anti‐CD4 and anti‐CD49d Abs. Peritoneal B-1a cells were sorted after staining with anti-CD19, anti-CD5, and anti-CD11b Abs. The staining with anti-CD4 and anti-CD49d Abs. Peritoneal CD49d highCD4+ or CD49d lowCD4+ T cells were then analyzed using a FACSCanto II flow cytometer. To measure the serum levels of IgG upon NAD+ injection, 8-wk-old Cd49d+ mice were repeatedly injected i.p. with NAD+ at 3-d intervals for 15 d.

**Quantitative real-time PCR analysis**

For the real-time PCR analysis, total cellular RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA) from peritoneal Cd49d+ and Cd49d+ T cells and in vitro generated Th1 and Th2 cells. Next, cDNA was synthesized using the RT First Strand Kit (Qiagen). Real-time PCR mixtures contained cDNA template, gene-specific primers, and SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA). Thermocycling was performed in a 7800 Real-Time PCR System (Applied Biosystems, Grand Island, NY). Relative quantification was performed using the 2^-ΔΔCt method and β-actin as the endogenous control gene. The following quantitative RT-PCR (qRT-PCR) primers were used (forward, reverse): Ccr5, 5'-TGGAGGAGATACGAGCT-3'; CCL25, 5'-TGTAAGGGATTCCCTGCTGC-T'; CCL5, 5'-CCGGACACGATGGTGGTTT-3'; CCL7, 5'-CAGCTCCTAAAGACTGCT-3'; IL2, 5'-TACCCTCCAGAGGAAGGAGG-3'; IL7, 5'-CAACACAGGACGCCAAAGGACT-3'; IL10, 5'-GGTGGCAATACAAGGCTGC-3'; IL12, 5'-GTCGCGCGCTGATGACGCTA-3'; IL13, 5'-TCTGGCTAGTTGGTCATG-3'; JAK3, 5'-AAGAAGGACCCACCTCT-3'; JAK6, 5'-TGTCGGAGACTGCTCTT-3'; JAK8, 5'-CAAGATAGAACC-3'; IFN-γ, 5'-GAAATATAAGCGGTTCGCT-3'; IL12-β1, 5'-GACATGAAAACACCT-3'; IL23, 5'-CAACAGACGAGGCCGAAGATG-3'; TNF-α, 5'-GGGGGCGTGGACATATAAGCGGTTC-3'; Bcl6, 5'-GGGCCACGAGGTCAATGATG-3'; B2m, 5'-TTGGTATTTCAATGGTCTCTTG-3'; Cxcr3, 5'-GGTTGCAATACAAGGCTGC-3'; Cx3cr1, 5'-GTCGCGCGCTGATGACGCTA-3'; Tgfb1, 5'-AAGAAGGACCCACCTCT-3'; Tgfb3, 5'-TGTCGGAGACTGCTCTT-3'; Tgfb4, 5'-CAAGATAGAACC-3'; CX3cr1, 5'-GAAATATAAGCGGTTCGCT-3'; CXCR3, 5'-GACATGAAAACACCT-3'; CXCL10, 5'-GGGGGCGTGGACATATAAGCGGTTC-3'; CXCL13, 5'-GTCGCGCGCTGATGACGCTA-3'; and Actb, 5'-AGAATATAAGCGGTTCGCT-3'; 5'-AAGAAGGACCCACCTCT-3'.

**In vitro generation of Th1 and Th2 cells**

Sorted naive Cd49d+ T cells from the spleen of Cd49d+ mice (5 × 10⁶ cells/well) were primed with plate-bound 5 μg/ml anti-CD3 (BD Biosciences) and 2.5 μg/ml anti-CD28 (BD Biosciences) for 3 d. For Th1 cell polarization, cells were stimulated in the presence of 10 μg/ml anti-IL-4 neutralizing Ab (BioLegend) and 10 ng/ml IL-12 (ProSpec, East Brunswick, NJ). For Th2 cell polarization, cells were stimulated in the presence of 10 μg/ml anti-IFN-γ neutralizing Ab (BioLegend), 10 ng/ml anti-IL-4 neutralizing Ab (BioLegend), and 5 ng/ml IL-6 (ProSpec). At day 5, the cells were washed with PBS and used for qRT-PCR analysis.

**Transwell chemotaxis assay**

Transmigration assays were performed using the Transwell system. Briefly, 0.5–1 × 10⁶ peritoneal Cd49dlo‐Cd49d+ T cells, peritoneal Cd49dlo‐Cd49d+ T cells, or splenic Cd49dlo‐Cd49d+ T cells sorted from Cd49d+ mice were loaded into the upper chamber of a Transwell device (5 μm pore size; Corning Costar, Cambridge, MA). The lower wells were filled with either 600 μl medium alone, or 600 μl 100 ng/ml CXCL9 (BioLegend), CXCL10 (eBioscience), or CXCL13 (BioLegend) in medium. The total numbers of cells that had migrated into the lower chambers after a 2-h incubation were then determined by flow cytometry.

**ELISAs**

The serum and cell culture levels of total IgM, IgG1, IgG2a, and IgG3 IgGs were determined by ELISAs. For in vitro coculture experiments, sorted Cd49d+ T cell subpopulations (5 × 10⁶ cells/well) from Cd49d+ or Sh2d2alow mice were cocultured with sorted peritoneal B1a cells or splenic B cells (5 × 10⁶ cells/well) in the presence of 0.5 μg/ml CD3-specific mAbs for 3 d. In these experiments, cells were transferred from donor to recipient. For adoptive cell transfer experiments, sorted peritoneal B1a cells were i.p. transferred into Rag1−/− mice, either with or without sorted peritoneal Cd49dlo or Cd49dw‐Cd49d+ T cells, or with splenic Cd49dlo‐Cd49d+ T cells. The IgM
concentrations in the sera from adoptively transferred Rag1<sup>−/−</sup> mice were then measured at indicated days.

To determine the amount of IFN-γ secreted by CD4<sup>+</sup> T cells, sorted CD4<sup>+</sup> T cell subpopulations (5 × 10<sup>4</sup> cells/well) were cultured in complete culture medium, either with or without 10 ng/ml IL-12 (ProSpec) for 2 d.

**Statistical analysis**

Student's <i>t</i> test (unpaired) and one-way or two-way ANOVA tests were used to assess the statistical significance of differences between groups. The <i>p</i> values <0.05 were considered to be statistically significant for all tests. Histograms were plotted using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

**Results**

**Conjugate formation between CD4<sup>+</sup> T and B-1a cells in the peritoneal cavity**

To identify the population of follicular Th (T<sub>FH</sub>)-like cells that support B-1 cell-mediated production of natural Abs, we first looked for peritoneal CXCR5<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 1A). There were a substantial number of CXCR5<sup>+</sup>CD4<sup>+</sup> T cells in the peritoneal cavity, but not in the spleen (Supplemental Fig. 1A), but most of them were shown to be conjugates of CD4<sup>+</sup> T cells and B cells, as revealed by both FSC-height versus FSC-area plots and their expression of both BCRs and TCRs (Fig. 1B). Despite a large individual variation, ∼3-6% of peritoneal CD4<sup>+</sup> T cells were in doublets with B cells, and the proportions of B–CD4<sup>+</sup> T cell doublets among total CD4<sup>+</sup> T cells were consistently higher in the peritoneal cavity than those in the spleen of the naive mice, which were ∼1-4% (Supplemental Fig. 1B). Next, the peritoneal B–CD4<sup>+</sup> T cell conjugates were sorted and observed by immunofluorescence microscopy, which were imaged as closely apposed B and CD4<sup>+</sup> T cells as doublets or triplets with the clustering of CD19 in the contact area (Fig. 1C). Then, sorted conjugates were separated and reanalyzed by flow cytometry. The conjugates were shown to be composed mainly of CD5<sup>+</sup> B–1a–CD4<sup>+</sup> T cell conjugates, because the separated B cells expressed both CD5 and CD11b as compared with singlet B cells (Fig. 1D). The ratio of CD4<sup>+</sup> T cells to B-1a cells was estimated to be ∼1:2, suggesting that many conjugates were multispots composed of single CD4<sup>+</sup> T cell and a few B cells. These findings imply that peritoneal CD4<sup>+</sup> T cells may provide helper functions for B-1a cells.

**Abundance of effector memory phenotype CD49d<sup>high</sup>CD4<sup>+</sup> T cells in the peritoneal cavity**

Because we thought that un conjugated peritoneal CD4<sup>+</sup> T cells could be memory/effector CD4<sup>+</sup> T cells capable of providing B-1a cell help before conjugation, the expression levels of memory/effector T cell markers were investigated in the peritoneal CD4<sup>+</sup> T cells. To our surprise, about half of the un conjugated peritoneal CD4<sup>+</sup> T cells expressed a high level of CD49d (α<sub>4</sub> integrin) (Fig. 2A). The proportion of CD49d<sup>high</sup>CD4<sup>+</sup> T cells was especially high in the peritoneal cavity, but not in other lymphoid tissues (Fig. 2C). When the total numbers of CD49d<sup>high</sup>CD4<sup>+</sup> T cells were calculated in the various lymphoid organs, the peritoneal cavity was estimated to contain a considerable fraction of the total CD49d<sup>high</sup>CD4<sup>+</sup> T cells in the whole body, considering that only some fraction of the peritoneal cells can be collected. Furthermore, splenic CD49d<sup>high</sup>CD4<sup>+</sup> T cells were phenotypically different from the peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells with respect to cell surface marker profiles and cytokine secretion, as described below (Fig. 3). Bone marrow CD4<sup>+</sup> T cells exhibited only a single peak of CD49d expression, rather than a bimodal expression pattern (data not shown). This abundant peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cell population was also detected in BALB/c mice (data not shown). The peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells constitutes ∼40% of all CD4<sup>+</sup> T cells conjugated to B cells (Fig. 1D). Because peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells were present in CD1d-deficient mice, these cells are not NKT cells (Fig. 2A). Furthermore, the proportion of peritoneal CD4<sup>+</sup> T cells conjugated to B cells was ∼5-6% of total CD4<sup>+</sup> T cells in the CD1d-deficient mice, similar to that in WT mice (Supplemental Fig. 1B). To exclude NKT cells that have the features of memory/effector T cells, we obtained cells from CD1d<sup>−/−</sup> mice for the majority of the subsequent experiments.

We next compared the expression levels of various activation and differentiation markers in peritoneal and splenic CD4<sup>+</sup> T cell subsets from CD1d<sup>−/−</sup> and WT mice. Compared with peritoneal and splenic CD49d<sup>low</sup>CD4<sup>+</sup> T cells, peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells showed an effector memory CD44<sup>high</sup>CD62L<sup>low</sup> phenotype (Fig. 2D, Supplemental Fig. 2A). Noticeably, the high level of CXCR3 expression in peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells suggests that these cells are able to enter peripheral inflammatory sites. To further characterize the peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells, we determined which integrin β-chain combined with CD49d in these cells. These experiments revealed that they preferentially expressed β<sub>1</sub> integrin, not α<sub>4</sub>β<sub>1</sub> integrin, suggesting that peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells can be categorized as effector memory CD4<sup>+</sup> T cells that can preferentially enter peripheral inflammatory sites via CXCR3 and VLA-4 (α<sub>4</sub>β<sub>1</sub> integrin). Meanwhile, peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells also showed high expression levels of CD1<sub>FH</sub> cell–related proteins, such as ICOS, programmed cell death 1, SLAM, CD122, and CD127 (Fig. 2D). Peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells from WT mice showed almost the same characteristics (Supplemental Fig. 2A). Interestingly, nearly all of peritoneal CD44<sup>high</sup>CD4<sup>+</sup> T cells showed a high expression of CD49d, whereas only ∼40% of splenic MP CD44<sup>high</sup>CD4<sup>+</sup> T cells were high for CD49d, with lower mean fluorescence intensity than that of peritoneal CD44<sup>high</sup>CD4<sup>+</sup> T cells (Fig. 2B). Collectively, these data indicate that peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells are a unique subpopulation of MP CD4<sup>+</sup> T cells, which possess characteristics of both proinflammatory memory Th1 cells and CD1<sub>FH</sub> cells.

**Rapid production of multiple Th1 cytokines by peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells**

Next, we investigated the cytokine profiles produced by the various CD4<sup>+</sup> T cell subsets from CD1d<sup>−/−</sup> mice. Upon stimulation of total peritoneal and splenic cells with PMA and ionomycin, gated peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells rapidly produced multiple Th1 cytokines, such as IFN-γ, TNF-α, and IL-2, within 4 h (Fig. 3). However, peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells did not produce IL-4, IL-5, or TGF-β, and a small proportion of peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells produced IL-10. In contrast, only a small proportion of the peritoneal and splenic CD49d<sup>low</sup>CD4<sup>+</sup> T cells secreted TNF-α and IL-2 upon PMA/ionomycin stimulation. Meanwhile, ∼9% of splenic CD49d<sup>high</sup>CD4<sup>+</sup> T cells secreted IFN-γ, TNF-α, and IL-2 upon PMA/ionomycin stimulation, which suggests that the peritoneal MP CD44<sup>high</sup>CD4<sup>+</sup> T cells were qualitatively different from splenic CD44<sup>high</sup>CD4<sup>+</sup> T cells. Similar proportion of splenic CD44<sup>high</sup>CD4<sup>+</sup> T cells secreted IFN-γ, TNF-α, and IL-2 upon PMA/ionomycin stimulation (Supplemental Fig. 3B). When sorted peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells from CD1d<sup>−/−</sup> mice were stimulated with PMA and ionomycin, very similar results were obtained (Supplemental Fig. 3C). The WT peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells similarly secreted IFN-γ, TNF-α, and IL-2 upon PMA/ionomycin stimulation (Supplemental Fig. 2B). These data indicate that peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells are Th1-biased MP CD4<sup>+</sup> T cells.

**Early development and maintenance of peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells**

Because peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells appear in naive WT and CD1d<sup>−/−</sup> mice raised in pathogen-free conditions, we inves-
tigated the development of these cells by determining whether they were present in young mice. Peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells were detected from 12 d of age (Fig. 4A). Next, we investigated whether peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells developed in the mouse strains with various genetic deficits. Peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells were found to be absent in BALB/c nude mice, which lack thymuses, and also in mice deficient in promoter IV of the MHC CIITA, which lack MHC class II expression on their cortical thymic epithelial cells (Fig. 4B). Furthermore, the development of peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells was independent of the expression of SLAM-associated protein (SAP), as these cells persisted in SAP-deficient mice that were known to be deficient in NKT cells (18). The SAP-deficient peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells were also able to rapidly secrete Th1 cytokines (data not shown). These data indicate that peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells may develop within the thymus, and that the recognition of MHC class II molecules on the surface of

**FIGURE 1.** Identification of CD4<sup>+</sup> T cells conjugated to B-1a cells in the peritoneal cavity. (A) Peritoneal cells obtained from 8-wk-old C57BL/6 mice were stained with fluorescently labeled anti-CXCR5 and anti-CD4 Abs. Gated CXCR5<sup>+</sup>CD4<sup>+</sup> T cells and CXCR5<sup>-</sup>CD4<sup>+</sup> T cells are shown according to FSC-height and FSC-area (upper panel). Total peritoneal cells are shown for FSC-height and FSC-area, and singlet and multiplet CD4<sup>+</sup> T cells are shown for the expression of CXCR5 (lower panel). (B) Expression of IgM and TCR-β in the given populations. (C) Sorted peritoneal B-CD4<sup>+</sup> T cell conjugates were observed by the immunofluorescence microscopy. CD4; FITC, CD19; PE. (D) Sorted peritoneal CD19<sup>-</sup>CD4<sup>+</sup> conjugated cells were separated with 10 mM EDTA in PBS with 0.5% BSA. Separated cells were examined for the expression of CD5, CD11b, and CD49d. Data shown are from one experiment and are representative of three independent experiments.
thymic epithelial cells may contribute to their positive selection of the peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells (Fig. 4B).

We reasoned that if peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells develop spontaneously as MP cells, they might exhibit stem cell–like features (19). To test this hypothesis, we investigated the occurrence of peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells in 4-wk-old mice that had undergone thymectomy at 3 d of age (Fig. 4C). Remarkably, peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells were still present in the Cd1d<sup>−/−</sup> mice that had undergone neonatal thymectomy, whereas peritoneal CD49d<sup>lo</sup>CD4<sup>+</sup> T cells were almost completely absent (Fig. 4C). This finding is in contrast to the previous observation that NKT cells were abolished by the neonatal thymectomy (20). Self-renewing peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells could consistently produce Th1 cytokines upon stimulation with PMA and ionomycin (Fig. 4D). Moreover, this population did not exhibit a restricted TCR repertoire, in contrast to known innate T cells such as iNKT cells or mucosa-associated invariant T cells. The distribution of V<sub>β</sub>-chain usage among the peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells was widespread and was not significantly different from those observed in peritoneal and splenic CD49d<sup>lo</sup>CD4<sup>+</sup> T cells (Fig. 5).

Peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells are more sensitive to P2X<sub>7</sub> agonists, but less sensitive to irradiation, than peritoneal CD49d<sup>lo</sup>CD4<sup>+</sup> T cells

During our studies of activation-related molecules, peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells sometimes exhibited a bimodal expression pattern of CD27, which was different from the expression pattern observed in peritoneal CD49d<sup>lo</sup>CD4<sup>+</sup> T cells (data not shown). We suspected that peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells might be susceptible to extracellular ATP or NAD<sup>+</sup> generated during in vitro cell preparation (21). Upon adding 100 μM ATP for 15 min in a short-term culture, we observed significantly greater downregulation of CD27 in the peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells compared with the peritoneal and splenic CD49d<sup>lo</sup>CD4<sup>+</sup> T cells (Fig. 6A) (22). Peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells were also more susceptible to NAD<sup>+</sup>– or ATP-induced cell death than either peritoneal or splenic CD49d<sup>lo</sup>CD4<sup>+</sup> T cells, because cell surface exposure of phosphatidylserine was more strongly induced by 10 μM NAD<sup>+</sup> or 200 μM ATP in the peritoneal CD49d<sup>hi</sup>CD4<sup>+</sup> T cells compared with the peritoneal and splenic CD49d<sup>lo</sup>CD4<sup>+</sup> T cells (Fig. 6B). The susceptibility of CD49d<sup>hi</sup>CD4<sup>+</sup> T cells to NAD<sup>+</sup>-induced cell
death was further confirmed in vivo. The ratio of CD49d<sup>high</sup>CD4<sup>+</sup> T cells to CD49d<sup>low</sup>CD4<sup>+</sup> T cells was dramatically decreased 24 h after an i.v. injection of NAD<sup>+</sup> (Fig. 6C). Collectively, these data indicate that peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells are more sensitive to P2X<sub>7</sub>-dependent regulation than either peritoneal or splenic CD49d<sup>low</sup>CD4<sup>+</sup> T cells. However, peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells were more resistant to irradiation than peritoneal CD49d<sup>low</sup>CD4<sup>+</sup> T cells, as measured after a whole-body 12 Gy γ-irradiation (Fig. 6C). Interestingly, peritoneal CD49d<sup>high</sup>CD4<sup>+</sup> T cells proliferated more slowly upon stimulation with anti-CD3 and anti-CD28.
Abs compared with peritoneal and splenic CD49d low CD4+ T cells (Fig. 6D).

**Peritoneal CD49d high CD4+ T cells exhibit properties of both Th1 cells and TFH cells**

Considering our results showing their early development and rapid secretion of proinflammatory cytokines, we hypothesized that peritoneal CD49d high CD4+ T cells might be innate-like T cells similarly to iNKT cells, which secrete IFN-γ in response to IL-12 stimulation in a manner independent of their TCR specificity (23). We tested whether peritoneal CD49d high CD4+ T cells also secreted IFN-γ upon IL-12 stimulation, and found that they did produce IFN-γ upon IL-12 stimulation, whereas peritoneal and splenic CD49d low CD4+ T cells did not (Fig. 7A). This result suggests that peritoneal CD49d high CD4+ T cells also possess an innate proinflammatory function.

We next performed qRT-PCR analysis to quantify the expression levels of critical genes related to Th1 and TFH cells (Fig. 7B). Significantly higher expression levels of Cxcr5, Il21, Batf (basic leucine zipper transcription factor, activating transcription factor-like), Tbx21 (T-bet), and Prdm1 (Blimp-1) were observed in the peritoneal CD49d high CD4+ T cells compared with the peritoneal CD49d low CD4+ T cells. T-bet, rather than Eomes, has been shown to be the principal transcription factor that determines the Th1 characteristics exhibited by peritoneal CD49d high CD4+ T cells, similar to iNKT cells (24), which further substantiate the innate-like features of the peritoneal CD49d high CD4+ T cells. However, the peritoneal CD49d high CD4+ T cells did not express a NKT lineage–specific BTB/POZ–zinc

**FIGURE 4.** Early development of CD49d high CD4+ T cells in the peritoneal cavity. (A) Peritoneal cells isolated from Cd1d−/− mice at 12 d of age were examined for the expression of CD4 and CD49d. (B) Peritoneal cells obtained from BALB/c nude mice and C57BL/6 CIITA promoter IV−/− and SAP−/− mice were examined for the expression of CD4 and CD49d. The numbers next to boxes indicate the estimated total numbers of the peritoneal CD49d high CD4+ and CD49d low CD4+ T cells (×105). (C) Newborn mice were thymectomized at 3 d of age (D3Tx). At 4 wk of age, peritoneal cells were harvested and examined for the expression of CD4 and CD49d. (D) Peritoneal cells from D3Tx mice were stimulated in vitro in the presence of 50 ng/ml PMA and 1.5 mM ionomycin for 4 h. The expression of various cytokines on the cells was then examined. Data shown are from one of three independent experiments.

**FIGURE 5.** Diverse TCR Vb usage by peritoneal CD49d high CD4+ T cells. Peritoneal and splenic cells were harvested from 8-wk-old Cd1d−/− mice and then stained with Abs against CD4, CD49d, and various TCR Vb-chains to compare the frequencies of cells expressing each TCR Vb-chain. Bars show the percentages of use of each TCR Vb-chain in peritoneal CD49d high or CD49d low CD4+ T cells, or splenic CD49d low CD4+ T cells. Representative data from three independent experiments are shown.
finger–type transcription factor, PLZF (Supplemental Fig. 3D).

Furthermore, peritoneal CD49d high CD4+ T cells also expressed several genes implicated in T FH cell function, such as Cxcr5, Il21, and Batf (25).

For functional demonstration of peritoneal CD49d high CD4+ T cells, we performed Transwell migration assays to confirm the functional activity of CXCR5 (Fig. 7C). Peritoneal CD49d high CD4+ T cells exhibited only a weak response to CXCL13, the ligand for CXCR5. This finding suggests that only a small fraction of the peritoneal CD49d high CD4+ T cells responds to CXCL13, which may be associated with the expression of CXCR5 in a small proportion of them (Fig. 2D). Meanwhile, more than half of peritoneal CD49d high CD4+ T cells migrated toward CXCL9 or CXCL10, the ligands for CXCR3 (Fig. 7D). Collectively, these data indicate that peritoneal CD49d high CD4+ T cells possess some features of innate-like proinflammatory Th1 cells, but also some features of T FH cells.

**B cell helper capacity of peritoneal CD49d high CD4+ T cells**

Our data suggest that peritoneal CD49d high CD4+ T cells do not completely meet the definition of T FH cells (26). To compare the
The proportions of the migrated T cells out of the total cell inputs are shown. Data shown are representative of three experiments. 

### Materials and Methods

#### CD49dhighCD4+ T cells exhibit characteristics of both Th1 and T FH cells.

(A) IFN-γ secretion upon stimulation with IL-12. A total of $1 \times 10^5$ sorted peritoneal CD49dhighCD4+ T cells, peritoneal CD49dmidCD4+ T cells, or splenic CD49dmidCD4+ T cells from Cdl1d−/− mice was stimulated with IL-12 for 24 h at 37°C. The amounts of IFN-γ in the culture supernatants were then analyzed by ELISA. (B) Gene expression profiles in peritoneal CD49dhigh or CD49dmidCD4+ T cells. As positive controls, Th1 or T FH cells that were generated in vitro, as described in Materials and Methods, were used. Real-time PCR analysis was performed using $1 \times 10^5$ sorted cells from Cdl1d−/− mice or in vitro generated cells. The expression levels of various genes in CD49dhighCD4+ T cells compared with those in CD49dmidCD4+ T cells are shown. ***p < 0.001, **p < 0.01, *p < 0.05. (C and D) Transwell chemotaxis assay. A total of $1 \times 10^5$ sorted peritoneal CD49dhighCD4+ T cells, peritoneal CD49dmidCD4+ T cells, or splenic CD49dmidCD4+ T cells from Cdl1d−/− mice was resuspended in serum-free RPMI 1640 medium. The lower wells were filled with 600 μl medium, either with or without 100 ng/ml CXCL13 (C), CXCL9, or CXCL10 (D). The proportions of the migrated T cells out of the total cell inputs are shown. Data shown are representative of three experiments.

We interpreted that the peritoneal CD49dhighCD4+ T cells in the lymphocyte-replete mice had much stronger B-1a cell-supporting activity than other subsets of CD4+ T cells.

To further estimate the role of the peritoneal CD49dhighCD4+ T cells in the production of natural IgM Ab, we repeatedly injected the Cdl1d−/− mice i.p. with NAD+ at 3-d intervals for 15 d to deplete peritoneal CD49dhighCD4+ T cells completely and measured the serum levels of Igs. Whereas the serum levels of IgG1, IgG2a, and IgG3 were not different before and after the NAD+ injections, the serum IgM levels were significantly decreased upon the NAD+ injections (Fig. 8D). Collectively, these data suggest that peritoneal CD49dhighCD4+ T cells are capable of providing enhanced help for B-1 cells and probably class-switched memory B cells.

### Discussion

Depending on the nature of the specific pathogens that they encounter, naïve CD4+ T cells adopt different programs of cytokine production (27). In contrast to other types of polarization, differentiation into T FH cells is independent of Th1, Th2, Th17, and regulatory T cell differentiation programs, as T FH cells with features of other polarized T cells have been recognized (28, 29). In particular, considerable flexibility between Th1 and T FH cells has been reported; namely, Th1 cells have been demonstrated to increase their B cell lymphoma 6 (Bcl-6) T-bet ratio under limiting IL-2 conditions to become T FH-like cells (30). In this study, we demonstrated the early development of peritoneal MP CD4+ T cells that possess functional characteristics of both Th1 cells and T FH cells. Peritoneal MP CD4+ T cells appear to be innate-like MP CD4+ T cells that maintain the long-term Th1 memory phenotype and also
adopt some of the early T_{FH} differentiation program in the peritoneal milieu.

T_{FH} cells are a specialized subset of CD4^+ T lymphocytes that mediate B cell class switching recombination and affinity maturation in the germinal center during Ab responses (31). T_{FH} cells were originally described as CD4^+ T cells that localize to the germinal center via the expression of CXCR5 (32, 33); however, it was later shown that most activated CD4^+ T cells upregulate CXCR5, with only a small fraction of these cells remaining as T_{FH} cells and exhibiting high expression of Bcl-6 (26, 34). We thought that most of the singlet CXCR5^+CD4^+ T cells were activated CD4^+ T cells in the peritoneal environment, but not T_{FH} cells (Fig. 1A). T_{FH} cell differentiation requires the interaction of activated CD4^+ T cells with dendritic cells and B cells to drive the upregulation of Bcl-6 (26, 34). We thought that most of the singlet CXCR5^+CD4^+ T cells were activated CD4^+ T cells in the peritoneal environment, but not T_{FH} cells (Fig. 1A). T_{FH} cell differentiation requires the interaction of activated CD4^+ T cells with dendritic cells and B cells to drive the upregulation of Bcl-6 (26, 34). Fully polarized T_{FH} cells, also referred to as germinal center T_{FH} cells, provide specific help for the selection of high-affinity B cells; however, pre-T_{FH} cells, which can be defined as all predecessors of T_{FH} cells, also support Ab production by B cells, especially outside the germinal center (26). In this regard, it was remarkable that the extrafollicular Th cells in MRL^{lpr} autoimmune mice were shown to enhance B cell IgG production through IL-21 and ICOS (35). We observed that peritoneal CD49d^hi CD4^+ T cells could provide help for both B-1a cells and memory B cells in vitro and in vivo. Because inflammatory stimuli, which are lacking in the homeostatic peritoneal cavity, are known to promote the development of T_{FH} cells (36), we hypothesized that peritoneal CD49d^hi CD4^+ T cells could be pre-T_{FH} cells possessing Th1 characteristics that have yet to upregulate Bcl-6 and are capable of circulating and entering the inflammatory sites via CXCR3. It is noteworthy that human circulating T_{FH} cells expressing CXCR3 efficiently induced memory B cells, not naive B cells, to differentiate into plasma cells in response to influenza vaccination (37). In mice, T_{FH} cells with features of Th1 cells were generated from STAT3-deficient CD4^+ T cells that expressed a high level of T-bet and a low level of Bcl-6 (38). It can be hypothesized that the peritoneal CD49d^hi CD4^+ T cells do not provide all aspects of B cell help, but some of the distinct functions such as proliferation, survival, plasma cell differentiation, and adhesion (39).
B-1a cells are CD5+ Mac-1- B cells that develop early in the fetal liver, reside in the peritoneal cavity, and generate natural IgM Abs (2). The production of natural Abs by B-1a cells is usually thought to be independent of CD4+ T cells and instead to be regulated by innate immune stimuli (2, 3). However, some pieces of evidence support the hypothesis that B-1a cells are regulated by CD4+ T cells (4). Furthermore, B-1a cells are now known to be important APCs for CD4+ T cells (40, 41), suggesting an active interaction between B-1a cells and CD4+ T cells. Our data indicate that peritoneal CD49dhiCD4+ T cells are Tfh-like cells capable of enhancing natural B-1a cell Ab secretion. During the systemic immune responses, B-1a cells accumulate in the regional lymphoid tissues and promote the adaptive immunity by B-2 cells and T cells (42). Because B-1a cells have stronger capacities of Ag presentation, cytokine secretion, and migration upon inflammatory stimuli than B-2 cells (43–45), it is interesting to address whether the peritoneal CD49dhiCD4+ T cells are responsible for these functions of B-1a cells other than natural Ab production, linking immune responses by innate lymphocytes to those by B-2 cells and naive CD4+ T cells.

The development of peritoneal MP CD4+ T cells at such an early age is intriguing. Although we could not observe CD49dhiCD4+ T cells within the thymus, the development of these cells appeared to be dependent on the thymus and thymic epithelial MHC class II expression. Their early development and memory cell–like features suggest that the peritoneal CD49dhiCD4+ T cells may be a subset of innate T cells similar to NKT cells. However, there are developmental differences between the peritoneal CD49dhiCD4+ T cells and NKT cells besides their basic difference in the restricting elements—MHC class II and CD1d. Notably, the relatively late development of NKT cells is in contrast with the early development of the peritoneal CD49dhiCD4+ T cells as the neonatal thymectomy at the age of 3 d depletes NKT cells (20). The persistence of CD49dhiCD4+ T cells in SAP-deficient mice also suggests that their development is distinct from the SAP-dependent development of NKT cells and innate CD8+ T cells (46). Therefore, CD49dhiCD4+ T cells appear to obtain their unique characteristics in the periphery after their exit from the thymus and perform proinflammatory functions in the peritoneal cavity or possibly in the inflammatory sites upon CXCR3-mediated migration. At present, the nature of the Ags that drive their peripheral differentiation is not known. Based on their diverse TCR repertoire, CD49dhiCD4+ T cells seem to not be restricted to a narrow set of positively selecting ligands. We hypothesize that the peritoneal CD49dhi CD4+ T cells and NKT cells are innate T cells that have complementary roles in recognizing different types of Ags and stresses and providing help for different types of cells. Whereas iNKT cells provide help for marginal zone B cells through CD1d-mediated Ag presentation (47), CD49dhiCD4+ T cells may be more biased to provide help for B-1a cells.

MP CD4+ T cells have been shown to develop within the thymus and to be elevated in Ilk-deficient mice (15, 48). These thymus-derived innate MP CD4+ T cells may give rise to CD49dhiCD4+ T cells in the peritoneal environment. Another possible mechanism by which CD49dhiCD4+ T cells may develop could be homeostatic proliferation. For example, IL-7–independent rapid proliferation, referred to as endogenous proliferation, has been shown to generate memory/effector phenotype T cells in lymphopenic situations (49). Because newborns are in a physiological lymphopenic status, homeostatic proliferation may be one mechanism by which innate-like T cells are generated. Peritoneal CD49dhiCD4+ T cells showed self-renewing properties after neonatal thymectomy. However, upon in vitro stimulation, peritoneal CD49dhiCD4+ T cells responded with rapid secretion of cytokines rather than proliferation. We hypothesize that they may proliferate in a homeostatic manner similarly to NKT cells (50). Their high expression of IL-15Rα (CD127) may suggest that they also sustain their population by IL-15. Peritoneal CD49dhiCD4+ T cells were radioresistant, yet susceptible to P2X7-mediated cell death. Interestingly, these two characteristics have also been reported in memory T cells (51, 52). We postulate that these potentially dangerous peritoneal CD49dhiCD4+ T cells and NKT cells may be negatively regulated by extracellular ATP or NAD+, which can be generated under inflammatory conditions (53).

We also showed that peritoneal CD49dhiCD4+ T cells exert an inflammatory function that is not dependent on the TCR. In particular, peritoneal CD49dhiCD4+ T cells secreted IFN-γ upon stimulation with IL-12. IL-12 is produced by activated macrophages and plays a pivotal role in the initiation of the inflammatory response and the establishment of protective Th1 responses (54). This inflammatory function is one of the most important functions of memory CD4+ T cells and NKT cells (5, 23, 55). Therefore, peritoneal CD49dhiCD4+ T cells appear to participate in the local inflammatory process by providing IFN-γ and to regulate the inflammatory program via Th1 polarization (56, 57).

Our data showed that a population of innate-like CD49dhiCD4+ T cells is enriched in the peritoneal cavity. These cells acquired strong proinflammatory Th1 characteristics and also possess the capability of providing B cell help during their early development. Because these cells have several features in common with B-1 cells, we propose to call these T-1 CD4+ T cells, in accordance with the name of the B-1 cells and their B-1 cell helper activity. We postulate that peritoneal CD49dhiCD4+ T cells may be necessary for the early activation of the T cell compartment and B-1a cells upon acute pathogenic events requiring a diverse TCR repertoire, whereas innate T cells with canonical TCRs respond to designated Ags.

Acknowledgments

We thank Shiv Pillai (Ragon Institute of MGH, MIT and Harvard), Seong Hoe Park (Graduate School of Immunology, Seoul National University College of Medicine), and Ki-Young Lee (Sungkyunkwan University School of Medicine) for critical comments and discussion of the manuscript. We also thank the Laboratory Animal Research Center for help with animal care and procedural support and the Core Laboratory in Samsung Biomedical Research Institute for help with flow cytometric cell sorting.

Disclosures

The authors have no financial conflicts of interest.

References

Supplementary Figure 1. Peritoneal CD4⁺ T cells form conjugates with B cells more preferentially than splenic CD4⁺ T cells. (A) Splenic cells from 8-week-old C57BL/6 mice are shown for the expression of CD4 and CXCR5. Gated CXCR5⁺ CD4⁺ and CXCR5⁻ CD4⁺ T cells are revealed according to FSC-height and FSC-area (upper panel). Total peritoneal cells are shown for FSC-H and FSC-A and singlet and doublet CD4⁺ T cells are shown for the expression of CXCR5 (lower panel). (B) Peritoneal or splenic cells from wild type or CD1d⁻/⁻ mice were shown for the expression of CD4 and CD19 without gating on singlet cells. The percentages of CD4⁺ CD19⁺ doublets were shown above the gating boxes. Data (A, B) are from one representative experiment among five independent experiments.
Supplementary Figure 2. Peritoneal CD49d<sup>high</sup> CD4<sup>+</sup> T cells from C57BL/6 wild type mice are T<sub>H</sub>1-biased memory phenotype CD4<sup>+</sup> T cells similarly to those from Cdl1<sup>−/−</sup> mice. (A) The expression of various cell surface proteins are shown for gated splenic CD49d<sup>low</sup> CD4<sup>+</sup> T cells or peritoneal CD49d<sup>low</sup> or CD49d<sup>high</sup> CD4<sup>+</sup> T cells from 8-week-old C57BL/6 mice. (B) Peritoneal CD49d<sup>high</sup> or CD49d<sup>low</sup> CD4<sup>+</sup> T cells from 8-week-old C57BL/6 mice were stimulated <i>in vitro</i> with 50 ng/ml PMA and 1.5 mM ionomycin for 4 hours. Cells were fixed, permeabilized, stained with given anti-cytokine mAbs, and analyzed by flow cytometry. The numbers in dot plots indicate proportions of cells in individual quadrants. Data (A, B) are from one representative experiment among three independent experiments.
Supplementary Figure 3. Cytokine production by splenic and peritoneal CD4⁺ T cell subsets and the analysis of the PLZF expression in peritoneal CD4⁺ T cell subsets and thymic NKT cells. (A) The expression of various cell surface proteins in gated peritoneal CD49d^{high} CD4⁺ T cells (red, Pch) and splenic CD49d^{high} CD4⁺ T cells (blue, SpH). (B) Cytokine production by gated splenic CD44^{high} or CD44^{low} CD4⁺ T cells. Splenic cells from 8-week-old CD1d⁻/− mice were stimulated in vitro with 50 ng/ml PMA and 1.5 mM ionomycin for 4 hours, fixed, permeabilized, and stained with given anti-cytokine mAbs. Positive control mice are WT mice 2 weeks after the immunization with ovalbumin and complete Freund adjuvant. Proportions of cells in individual quadrants were shown. Data A and B are from one representative experiment among three. (C) Cytokine production by sorted peritoneal CD49d^{high} or CD49d^{low} CD4⁺ T cells or splenic CD49d^{low} CD4⁺ T cells. Cells were sorted from 8-week-old CD1d⁻/− mice and stimulated in vitro with 50 ng/ml PMA and 1.5 mM ionomycin for 4 hours. Cells were analyzed for the expression of given cytokines by flow cytometry. Data are from one of two independent experiments. (D) The expression of PLZF in gated peritoneal CD49d^{high} CD4⁺ T cells (solid red) and peritoneal CD49d^{low} CD4⁺ T cells (dotted) from CD1d⁻/− mice, thymic NK1.1⁺ CD4⁺ T cells (black filled) and thymic NK1.1⁺CD4⁺ T cells (gray filled) from C57BL/6 WT mice.

Supplementary Figure 4. The expression of CD49d on adoptively transferred peritoneal or splenic CD4⁺ T cells into Rag1⁻/− mice on day 54 after the transfer. Sorted peritoneal B1a cells were adoptively transferred with or without sorted peritoneal CD49d^{high} or CD49d^{low} CD4⁺ T cells, or splenic CD49d^{low} CD4⁺ T cells to Rag1⁻/− mice for the measurement of serum IgM antibodies as shown in Fig. 8C. On day 54 after the adoptive transfer, harvested peritoneal cells were stained with fluorescently labeled anti-CD4 and anti-CD49d antibodies.