

CUTTING EDGE

Cutting Edge: A Possible Role for CD4⁺ Thymic Macrophages as Professional Scavengers of Apoptotic Thymocytes¹Eiji Esashi,* Takashi Sekiguchi,* Hiroaki Ito,* Shigeo Koyasu,^{†‡} and Atsushi Miyajima^{2*‡}

A vast majority of thymocytes are eliminated during T cell development by apoptosis. However, apoptotic thymocytes are not usually found in the thymus, indicating that apoptotic thymocytes must be eliminated rapidly by scavengers. Although macrophages and dendritic cells are believed to play such role, little is known about scavengers in the thymus. We found that CD4⁺/CD11b⁺/CD11c⁻ cells were present in the thymus and that they expressed costimulatory molecules for T cell selection and possessed Ag-presenting activity. Moreover, these CD4⁺/CD11b⁺ cells phagocytosed apoptotic thymocytes much more efficiently than thymic CD4⁻/CD11b⁺ cells as well as activated peritoneal macrophages. CD4⁺/CD11b⁺ cells became larger along with thymus development, while no such change was observed in CD4⁻/CD11b⁺ cells. Finally, engulfed nuclei were frequently found in CD4⁺/CD11b⁺ cells. These results strongly suggest that thymic CD4⁺/CD11b⁺ cells are major scavengers of apoptotic thymocytes. The Journal of Immunology, 2003, 171: 2773–2777.

Macrophages play a major role in innate immunity by eliminating bacteria and other foreign substances by their phagocytic activity and also produce a battery of cytokines and chemokines to induce adaptive immunity. Another important role for macrophages is to eliminate apoptotic cells by phagocytosis. During T cell development in the thymus, T cells encounter APCs and are subjected to either positive or negative selection (1, 2). CD4⁺/CD8⁺ double-positive (DP)³ cells that recognize MHC on the surface of thymic stromal cells survive, whereas those DP cells that do not recognize MHC die by apoptosis. DP cells that respond to self-Ags are also depleted by apoptosis, and then DP cells differentiate to either CD4 or CD8 single-positive cells (3, 4). Besides T cells, the thymus contains fibroblasts, epithelial cells, dendritic cells (DCs), and macrophages. Thymic epithelial cells have been

shown to play an essential role for positive selection and DCs are believed to be involved in negative selection (5).

Although autoreactive T cells are depleted by apoptosis, apoptotic thymocytes are hardly found in the thymus under the normal developmental process. Thus, it was suggested that thymic macrophages eliminate apoptotic thymocytes (6, 7). During the apoptotic process, a cell autonomous DNA fragmentation is performed by caspase-activated DNase. However, T cell development was mostly intact in the caspase-activated DNase-deficient thymus. In contrast, a lack of DNase II, which is present in the lysosomes of macrophages, caused thymic involution (8), suggesting that DNA degradation by phagocytes is important for thymic development. In fact, mice with impaired phagocytosis exhibited splenomegally due to accumulation of massive apoptotic cells. Such defects in humans lead to retinitis pigmentosa (9). Thus, rapid elimination of apoptotic cells is important for preventing inflammatory and autoimmune responses against self-Ags. However, the origin and the characteristics of scavengers for apoptotic T cells in the thymus remain largely unexplored.

In this report, we demonstrate that there is a novel class of macrophage expressing CD4 and that these CD4⁺ thymic macrophages exhibit higher Ag-presenting activity than CD4⁻/CD11b⁺ cells. In addition, CD4⁺ thymic macrophages exhibit phagocytic activity significantly higher than CD4⁻/CD11b⁺ cells from the peritoneum as well as the thymus. Finally, engulfed nuclei were frequently found in CD4⁺/CD11b⁺ cells. Our results strongly suggest that CD4⁺ thymic macrophages play a major role for the elimination of apoptotic T cells in the thymus.

Materials and Methods

Mice

Timed pregnant C57BL/6 and BALB/c mice were purchased from Nihon SLC (Hamamatsu, Japan), RAG-1-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The time at mid-day was taken to be embryonic day (E) 0.5 for the plugged mice. All mice were housed in specific pathogen-free barrier animal facilities. All experiments were performed according to our institutional guidelines.

*Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan; [†]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan; and [‡]Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Kawaguchi, Japan

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² Address correspondence and reprint requests to Dr. Atsushi Miyajima, Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. E-mail address: miyajima@ims.u-tokyo.ac.jp

³ Abbreviations used in this paper: DP, double-positive; DC, dendritic cell; E, embryonic day; TGC, thioglycollate; Dex, dexamethasone; DAPI, 4',6'-diamidino-2-phenylindole; WT, wild type.

Flow cytometry and cell sorting

Thymi were disaggregated and filtrated through a 40- μ m mesh. Cells suspended in PBS were pretreated with blocking Ab against the Fc γ R (FcR; 2.4G2) to eliminate nonspecific staining. They were then incubated with primary Abs or appropriate isotype-matched control Abs. Primary Abs used were FITC-conjugated CD11b (FITC-CD11b), FITC-CD11c, FITC-CD40, FITC-CD80, FITC-CD86, PE-conjugated CD11b (PE-CD11b), PE-CD4, biotinylated anti-CD4 (biotin-CD4), biotin-MHC class II (IAb), biotin-ICAM (all from BD PharMingen, San Diego, CA), and FITC-F4/80 (BioSource International, Camarillo, CA). After a 30-min incubation, cells were washed with PBS. Cells were then incubated with streptavidin-allophycocyanin (Molecular Probes, Eugene, OR). After washing with PBS, cells were resuspended in PBS with propidium iodine and analyzed by FACSCalibur (BD Biosciences, Mountain View, CA). Dead cells were excluded by propidium iodine staining. Cell sorting was performed by FACS Vantage SE (BD Biosciences).

Mixed lymphocyte reaction

CD4⁺/CD11b⁺ cells, CD4⁻/CD11b⁺ cells, splenic DCs, and LPS-stimulated DCs were used as APCs. CD4⁺/CD11b⁺ cells and CD4⁻/CD11b⁺ cells were isolated from E14.5 C57BL/6 thymus by FACS Vantage SE (BD Biosciences). DCs were purified from C57BL/6 spleen by positive selection on a MACS column using mAb to CD11c. The purity of cells was >93% by FACS analysis. These cells were cocultured with 10⁵ naive CD4 T cells isolated from BALB/c mice. CD4 T cells were prepared from spleens and purified by positive selection on a MACS column using mAb to CD4. Over 98% of purified cells were positive for CD4. Purified T cells (10⁵) were cultured with gamma ray-irradiated (30 Gy) purified APCs for 5 days. T cell proliferation was measured using the WST-1 system (Roche, Basel, Switzerland) and the OD_{450/650} was measured after 4 h by a microplate reader.

Preparation of peritoneal macrophages

C57BL/6 mice received an i.p. injection of 3% (w/v) thioglycollate (TGC) or were left untreated. After 4 days, ice-cold PBS was injected into the peritoneum, and the peritoneal fluid was recovered. The cells were resuspended in the culture medium and were plated in culture dishes. After a 2-h incubation, nonadherent cells were removed by extensive washing with PBS. Attached cells were used as peritoneal macrophages.

Phagocytosis assay

CD4⁻/CD11b⁺ and CD4⁺/CD11b⁺ cells were isolated from E14.5 thymus by FACS Vantage. For detection of latex bead incorporation, the cells were resuspended in the culture medium with a suspension of carboxylate-modified green fluorescent latex beads at a 0.05% dilution (1.1- μ m diameter; Sigma-

Aldrich, St. Louis, MO). The cells were plated in parmanox chamber slides (Nalge Nunc International, Rochester, NY) and incubated at 37°C for 2 h. After extensive washing three times, the cells were then fixed with 4% paraformaldehyde in PBS (4% PFA/PBS). After fixation, macrophages were stained with PE-CD11b and analyzed under a fluorescent microscope. For induction of apoptosis, thymocytes were incubated at 37°C for 12 h with 10⁻⁷ M dexamethasone (Dex) in the culture medium. Apoptotic induction was confirmed by TUNEL and annexin V staining (data not shown). After 12 h, thymocytes were incubated with 100 ng/ml 4',6'-diamidino-2-phenylindole (DAPI) for 15 min at 4°C. These cells were used for phagocytosis assays. Apoptotic thymocytes (4 \times 10⁶ cells) were added to macrophages on eight-well parmanox chamber slides. Phagocytosis was allowed to proceed as described above. After fixation, macrophages were stained with PE-CD11b. Phagocytosis was evaluated by microscopic observation.

Results

CD4⁺/CD11b⁺ cells in thymus

Flow cytometric analysis of fetal thymocytes revealed the presence of a distinct population of cells expressing both CD11b and CD4 in the thymus in addition to the CD4⁻/CD11b⁺ cells (Fig. 1A). All of these CD4 and CD11b DP cells also expressed F4/80, and were mostly negative for CD11c. In contrast, CD4⁻/CD11b⁺ cells were positive for CD11c (Fig. 1C). Although CD4⁺/CD11b⁺ cells were a small population (~1%) in the fetal thymus, they were found not only in the fetal thymus, but also in the adult thymus (see Fig. 5C). Freshly isolated CD4⁺/CD11b⁺ cells showed macrophage morphology with vacuoles and CD4⁻/CD11b⁺ cells exhibited the phenotype of immature DCs (Fig. 1B), suggesting that CD4⁺/CD11b⁺ cells are macrophages and CD4⁻/CD11b⁺ cells are mainly DCs. RT-PCR analysis showed that they did not express DC cytokines (Fig. 2A). Interestingly, CD4⁺/CD11b⁺ cells expressed costimulatory molecules such as CD80 and CD86 (Fig. 1C). Indeed, freshly isolated CD4⁺/CD11b⁺ cells stimulated proliferation of naive allogenic T cells (Fig. 2). These results indicate that CD4⁺/CD11b⁺ cells possessed Ag-presenting activity.

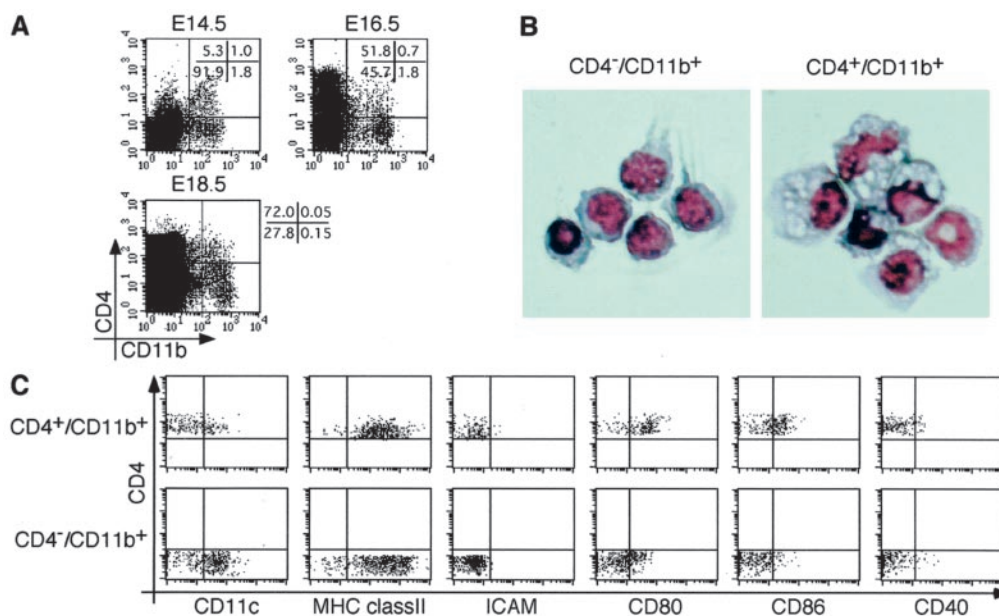


FIGURE 1. CD4⁺ macrophages in the thymus. *A*, Thymocytes obtained from E14.5, E16.5, and E18.5 thymi were stained with Abs against CD11b and CD4, and analyzed by flow cytometry. *B*, Morphology (Giemsa staining of cytospun cells) of CD4⁺/CD11b⁺ cells freshly isolated from E14.5 thymus. *C*, CD4⁺/CD11b⁺ cells from E14.5 thymus were analyzed by flow cytometry. CD4⁺/CD11b⁺ cells were negative for CD11c and expressed costimulatory molecules.

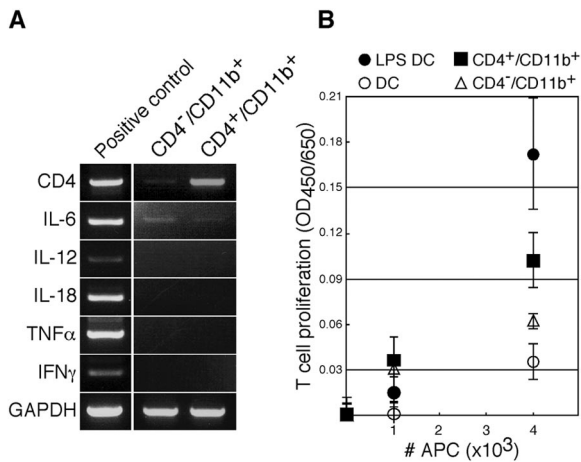


FIGURE 2. Ag-presenting activity of $CD4^+$ thymic macrophages. *A*, Cytokine expression of $CD4^+/CD11b^+$ cells. RNA was recovered from freshly isolated $CD4^+/CD11b^+$ cells and RT-PCR was performed. LPS-stimulated splenocytes were used as positive controls. *B*, Proliferation of naive allogenic T cells incubated with the indicated numbers of freshly isolated APCs. Experiments were preformed in triplicate (mean \pm SD).

Phagocytic activity of $CD4^+$ thymic macrophages

Most of the T cells are eliminated during development by apoptosis, while apoptotic T cells are hardly found in the thymus, indicating that there must be scavengers for apoptotic T cells in the thymus. Therefore, we considered the possibility that $CD4^+/CD11b^+$ cells are scavengers of apoptotic thymocytes. To confirm whether the $CD4^+/CD11b^+$ cells are macrophages with phagocytic activity, we isolated this population from the thymus and examined their phagocytic activity. The $CD4^+/CD11b^+$ cells freshly isolated from the thymus phagocytosed latex beads much more efficiently than $CD4^-/CD11b^+$ cells from the thymus (Fig. 3). We then performed phagocytic assays using apoptotic thymocytes induced by Dex treatment. The $CD4^+/CD11b^+$ cells prepared freshly from the thymus phagocytosed apoptotic thymocytes much more efficiently than

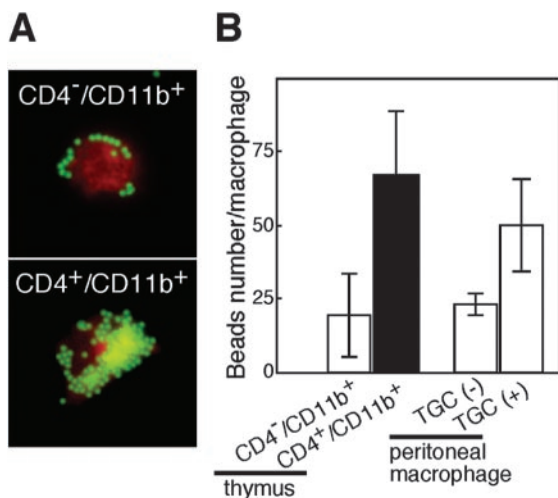


FIGURE 3. Bead incorporation of $CD4^+$ thymic macrophages. *A*, $CD4^-/CD11b^+$ and $CD4^+/CD11b^+$ cells from E14.5 thymus were cultured with FITC-conjugated latex beads and the cells were stained with anti-CD11b Ab (red). Their phagocytic activity was analyzed under a fluorescent microscope. *B*, The efficiency of phagocytosis was evaluated by counting latex beads incorporated in the cells. Error bars indicate SD.

$CD4^-/CD11b^+$ cells (Fig. 4*A*). Phagocytic activity of different macrophage populations was compared by counting the apoptotic nuclei engulfed in the phagocytes. Peritoneal macrophages were $CD4^-$ (data not shown) and exhibited phagocytic activity for apoptotic thymocytes. TGC, an activator of peritoneal macrophages, increased the phagocytic activity of peritoneal macrophages (Fig. 4*B*). Dex-untreated healthy cells were not phagocytosed by macrophages (data not shown). $CD4^-/CD11b^+$ cells in the thymus phagocytosed apoptotic thymocytes at a level similar to unstimulated peritoneal macrophages. Interestingly, $CD4^+/CD11b^+$ cells incorporated apoptotic cells much more efficiently (as much as 6-fold) than $CD4^-/CD11b^+$ thymic cells (Fig. 4*B*). These results suggest that $CD4^+/CD11b^+$ cells are more professional scavengers for apoptotic cells in the thymus.

$CD4^+$ thymic macrophages and T cell development

If $CD4^+/CD11b^+$ cells are professional scavengers for apoptotic T cells, we supposed that $CD4^+/CD11b^+$ cells should become larger along with T cell development in the fetal thymus as they are expected to engulf many apoptotic T cells. Therefore, we compared the size of $CD4^+/CD11b^+$ cells in different stages of development. It is well known that T cell selection starts at around E16. Consistent with the T cell development, the size of $CD4^+/CD11b^+$, but not $CD4^-/CD11b^+$, cells was significantly increased (Fig. 5*A*). In contrast, no such size change of $CD4^-/CD11b^+$ cells was observed in later stages of fetal thymus (Fig. 5*B*). The possibility that $CD4^+/CD11b^+$ cells are scavengers for apoptotic thymocytes was further supported by RAG-1-deficient mice, in which T cell selection does not occur. As RAG-1-deficient mice lack apoptotic T cells due to the defective T cell development, the size of $CD4^+/CD11b^+$ cells in RAG-1-deficient thymus is expected to remain small. We compared cell size between $CD4^+/CD11b^+$ and $CD4^-/$

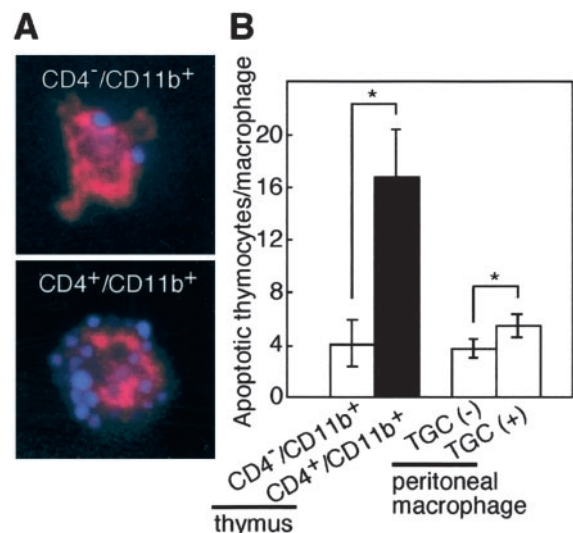


FIGURE 4. Efficiency of phagocytosis. *A*, Apoptotic thymocytes were stained with DAPI (blue) and then incubated with $CD4^-/CD11b^+$ or $CD4^+/CD11b^+$ cells from E14.5 thymus. The cells were then stained with anti-CD11b Ab (red), and analyzed under a fluorescent microscope. *B*, Phagocytosis was examined in each freshly isolated macrophages by a fluorescent microscope and the efficiency of phagocytosis was evaluated by apoptotic nuclei incorporated in the macrophages. The numbers of DAPI-positive cells per macrophage are shown. Error bars indicate SD; *, significant differences, $p < 0.01$.

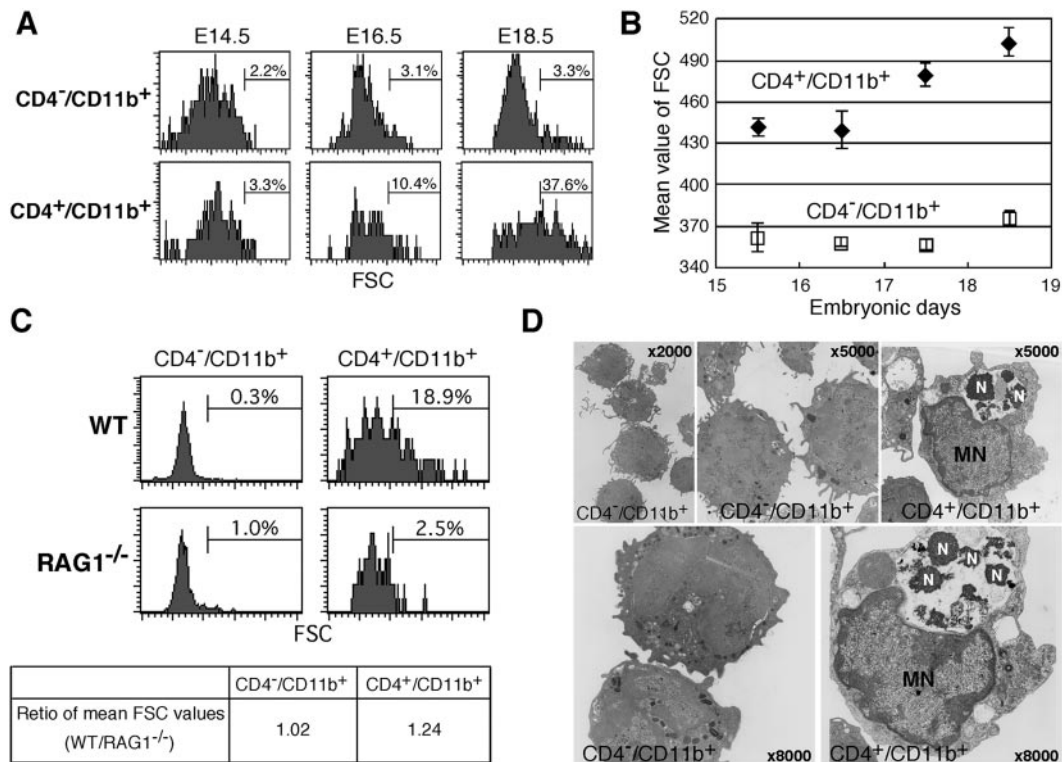


FIGURE 5. Cell size change of CD4⁺ macrophages during the development of the thymus. *A* and *B*, Comparison of cell size between CD4⁻/CD11b⁺ cells and CD4⁺/CD11b⁺ cells at each developmental stage. Note that CD4⁺/CD11b⁺ cells become bigger along with development. *B*, Experiments were performed over three times at each embryonic day. Error bars (SD) mean each experimental difference, but not each cell size difference. *C*, Comparison of cell size between WT and RAG-1-deficient adult thymus. WT and RAG-1^{-/-} mice used were littermates. *D*, Nucleus-like structures in CD4⁺/CD11b⁺ cells. CD4⁺/CD11b⁺ cells or CD4⁻/CD11b⁺ cells freshly isolated from E18.5 thymus were analyzed by electron microscopy. N, nucleus-like structure; MN, macrophage nucleus.

CD11b⁺ cells in both the wild-type (WT) and RAG-1-deficient thymus. In CD4⁻/CD11b⁺ cells, the cell size was not different between WT and RAG-1^{-/-}. In contrast, large CD4⁺/CD11b⁺ cells were found only in WT, but not RAG-1^{-/-} (Fig. 5C). We finally examined freshly isolated CD4⁺/CD11b⁺ cells from the thymus by transmission electron microscopy. A few nucleus-like structures, presumably engulfed nuclei, were frequently found in CD4⁺/CD11b⁺ cells but not in CD4⁻/CD11b⁺ cells (Fig. 5D); engulfed nuclei were found in as much as 40% of CD4⁺/CD11b⁺ cells whereas only 3% of CD4⁻/CD11b⁺ cells contained such nuclei. This result suggests that apoptotic cells were preferentially engulfed by CD4⁺/CD11b⁺ cells in the thymus. These results collectively suggest that thymic CD4⁺/CD11b⁺ cells are major scavengers of apoptotic thymocytes.

Discussion

In this report, we demonstrated that CD4⁺/CD11b⁺ cells were present in the thymus and had Ag-presenting activity and exhibited much stronger phagocytic activity than activated peritoneal macrophages. CD4⁺/CD11b⁺ cells exhibited ~6-fold higher phagocytic activity for apoptotic thymocytes than TGC-activated macrophages (Fig. 4B), which are known to engulf apoptotic cells efficiently (10). We also showed that engulfed nuclei were detected only in thymic CD4⁺/CD11b⁺ cells but not in CD4⁻/CD11b⁺ cells. Although a vast majority of thymocytes die by apoptosis during thymic selection, the population of potential scavengers such as macrophages and DCs is very small in the thymus (~0.1% of total thymic cells). There-

fore, our results strongly suggest that CD4⁺/CD11b⁺ cells are engaged in elimination of apoptotic T cells in the thymus as professional scavengers.

Among various types of blood cells, the DC is closely related to macrophages functionally and developmentally. Myeloid and lymphoid DCs play distinct roles for immune reaction. It was also reported that DCs in the thymus (thymic DCs) might play a key role for depletion of autoreactive T cells (11). Although DCs were considered to be generated from myeloid progenitors, it was shown that thymic DCs are derived from intrathymic lymphoid progenitors (12, 13). Myeloid DCs present Ags to T cells and are essential for the initiation of primary T cell responses. They also regulate the nature and extent of the immune responses by producing cytokines and chemokines (14). In contrast, lymphoid DCs are involved in the establishment of self-tolerance including negative selection in the thymus. It was also reported that thymic involution was caused by inflammatory cytokines. Thus, it is suggested that DCs and macrophages in the thymus might not produce these cytokines and our results support this possibility (Fig. 2A). In addition to the distinct characteristics of CD4⁺/CD11b⁺ cells in the thymus as described above, it is of interest to know the origin of these macrophages. Although macrophages are believed to be generated from myeloid progenitors, it was recently reported that macrophages in the lymph node and spleen might be derived from lymphoid progenitors (15) and that thymocytes differentiated into macrophages *in vitro* (16, 17). Thus, it is possible that CD4⁺/CD11b⁺ cells, like thymic DCs, might be originated from a distinct class of progenitors in the thymus.

While definitive roles and origins of CD4⁺ macrophages await further investigation, it is concluded that CD4⁺/CD11b⁺ cells are a novel class of macrophages with strong phagocytic activity and possibly function as professional scavengers of apoptotic T cells in the thymus.

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