Development of CD4+ Macrophages from Intrathymic T Cell Progenitors Is Induced by Thymic Epithelial Cells

Eiji Esashi, Hiroaki Ito, Katsuhiko Ishihara, Toshio Hirano, Shigeo Koyasu and Atsushi Miyajima

J Immunol 2004; 173:4360-4367; doi: 10.4049/jimmunol.173.7.4360
http://www.jimmunol.org/content/173/7/4360

References
This article cites 37 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/173/7/4360.full#ref-list-1

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
Development of CD4+ Macrophages from Intrathymic T Cell Progenitors Is Induced by Thymic Epithelial Cells

Eiji Esashi,* Hiroaki Ito,* Katsuhiko Ishihara,† Toshio Hirano,‡‡ Shigeo Koyasu,§∥ and Atsushi Miyajima*†∥

It was recently demonstrated that there are CD4+ macrophages, which exhibit strong phagocytic activity, in the thymus. They are suggested to play an important role for the elimination of apoptotic thymocytes. However, the origin and nature of CD4+ macrophages in the thymus remain unexplored. In this study, we describe that the most immature intrathymic progenitors (CD25+/CD44+/Fcer+) give rise to CD4+ macrophages by oncostatin M-responsive thymic epithelial cells (ORTEC) in an IL-7-dependent manner. Neither conditioned medium of ORTEC nor a mixture of cytokines induced CD4+ macrophages, and oncostatin M receptor was not expressed in thymocytes, suggesting that the development of CD4+ macrophages from the immature thymocytes requires a direct interaction with ORTEC. These results collectively suggest that the development of CD4+ macrophages from the intrathymic T cell progenitors is induced by thymic epithelial cells. The Journal of Immunology, 2004, 173: 4360–4367.

T cell development occurs in the thymus. CD4−/CD8− (double negative, DN) immature T cell progenitors become CD4+CD8+ (double positive, DP) cells with a TCR, which then undergo either positive or negative selection (1, 2). DP cells survive when their TCRs bind weakly to self-MHC molecules on the surface of thymic stromal cells, whereas those DP cells whose TCRs do not recognize self MHC die by apoptosis. In contrast, DP cells, whose TCRs bind strongly to self-Ags in association with self-MHC, are depleted by apoptosis and then DP cells differentiate to either CD4+ or CD8+ single-positive cells (3, 4). Thymic stromal cells, which consist of epithelial cells, dendritic cells, and macrophages, play specific roles for T cell development at different stages (5).

Dendritic cells (DCs) are major APCs essential for the activation of naive T cells and the initiation of immune responses (6). Moreover, it was reported that DCs in the thymus (thymic DCs) might play a key role for depletion of autoreactive T cells (7). While it was initially considered that DCs were generated from myeloid progenitors, it was later shown that thymic DCs are derived from intrathymic lymphoid progenitors, common precursors of T and NK cells (8, 9). Macrophages are present in various tissues including the thymus and phagocytose microbes and apoptotic cells. Because a vast majority of T cells are depleted during T cell selection, thymic macrophages are suggested to be scavengers for apoptotic thymocytes (10, 11). Although macrophages are generated from myeloid progenitors, it was also reported that macrophages in the lymph node might be derived from lymphoid progenitors (12), and thymocytes could differentiate into macrophages in vitro (13, 14).

Thymic epithelial cells (TECs) are involved in the T cell selection by the direct interaction with thymocytes and are composed of two major subsets, cortical and medullary epithelia, which are distinguished by the expression of keratin 8 and keratin 5, respectively (15). It was recently reported that the interaction of thymocytes with TECs is important for the differentiation of TECs. Thymocytes regulate TECs to build up the thymic architecture, resulting in the formation of cortical microenvironments followed by the establishment of medullary microenvironments (15, 16). Although heterogeneity of TECs has been recognized, little is known about the nature of each subpopulation of TECs.

T cell development is also supported by cytokines produced from thymic stromal cells. TECs support T cell development by production of cytokines and chemokines that regulate the proliferation and migration of thymocytes in a differentiation-dependent manner (17). T cell progenitors and other lymphoid progenitors require IL-7 for their survival and proliferation (18, 19). Oncostatin M (OSM) is a member of the IL-6 family of cytokines and its receptor consists of the OSM specific receptor subunit (OSMRβ) and gp130, the common receptor subunit of this cytokine family. It was reported that OSM is expressed in CD45-positive hematopoietic cells, whereas OSMRβ is not expressed in most of CD45-positive cells (20). In the thymus, OSMRβ expression was detected from fetus to adult (21). Expression of OSM by the lck proximal promoter in transgenic mice resulted in thymic hypertrophy, extrathymic T cell development, and autoimmune disease-like symptoms (22, 23). These results suggest that OSM may play a role for thymic environment.
Most T cells are eliminated during development by apoptosis, while apoptotic T cells are hardly found in the normal thymus, indicating that they must be eliminated quickly by scavengers in the thymus. We recently found a novel class of thymic macrophages with CD4, which exhibit strong phagocytic activity, suggesting that the CD4 macrophages are involved in the T selection by eliminating apoptotic T cells (24). However, the origin and nature of CD4 macrophages in the thymus remain unexplored. In this report, we demonstrate that a subpopulation of TECs with OSMR as well as the OSM-responsive TEC line (ORTEC), but not soluble factors, induces the development of CD4 macrophage from intrathymic lymphoid progenitors in an IL-7-dependent manner.

Materials and Methods

Mice

Timed pregnant C57BL/6 mice were purchased from Nihon SLC (Hamamatsu, Japan). gp130/D mice used in this paper were backcrossed with C57BL/6 eight times. The time at midday was taken to be E0.5 for the plugged mice. All mice were housed in specific-pathogen-free animal facilities. All experiments were performed according to our institutional guidelines.

Generation of mAb against murine OSMR (mOSMR)

Wistar rats (Nihon SLC) were immunized with recombinant soluble mOSMR by the standard immunization procedure (25). Lymph nodes were dissociated and fused with mouse myeloma P3X cells as previously described (26), and hybridoma supernatants were screened for the production of anti-mOSMR specific Abs by flow cytometry. Anti-mOSMR Ab (E26-2A8) was selected and was produced in nude mice. The isotype of anti-mOSMR Ab was determined as IgG1 by using the rat IgG isotyping kit (Serotec, Oxford, U.K.).

Flow cytometry and cell sorting

Cells suspended in PBS were pretreated with blocking Ab against the FcγR (FcγR 2/4G2) for 30 min on ice to eliminate nonspecific binding. They were then incubated with primary Abs or appropriate isotype-matched control Abs. Primary Abs used were FITC-conjugated anti-murine CD11b (FITC-CD11b), FITC-CD11c, FITC-CD40, FITC-CD80, FITC-CD86, FITC-ICAM PE-conjugated CD11b (PE-CD11b), PE-CD11c, PE-MHC class II, biotinylated anti-murine CD4 (biotin-CD4), biotin-CD11b, FITC-CD11c, FITC-CD4, FITC-CD80, FITC-CD86, FITC-ICAM PE-conjugated CD11b (PE-CD11b), PE-CD11c, PE-MHC class II, biotinylated anti-murine biotin-CD4 (biotin-CD4), biotin-CD11b, biotin-CD25, biotin-CD45 (all from BD Pharmingen, San Diego, CA), and FITC-Flk-1 (BioSource International, Camarillo, CA). After 30 min of incubation, cells were washed with PBS. Cells were then incubated with streptavidin-allophycocyanin (Molecular Probes, Eugene, OR) for 30 min on ice and washed with PBS. After washing, cells were resuspended in 50 μl of PBS with propidium iodine and analyzed by FACScalibur (BD Biosciences). The recovered fetal thymic macrophages with CD4, which exhibit strong phagocytic activity, suggested that CD4 macrophages are involved in the T selection by eliminating apoptotic T cells (24). However, the origin and nature of CD4 macrophages in the thymus remain unexplored. In this report, we demonstrate that a subpopulation of TECs with OSMR as well as the OSM-responsive TEC line (ORTEC), but not soluble factors, induces the development of CD4 macrophage from intrathymic lymphoid progenitors in an IL-7-dependent manner.

Table 1. Primer sets used for PCR amplification

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense</th>
<th>Antisense</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSM</td>
<td>5'-TCGGCCCTCAAAACCTGAGA-3'</td>
<td>5'-ATGTTATCCCGAGGAGAC-3'</td>
<td>56</td>
</tr>
<tr>
<td>OSMR</td>
<td>5'-ACTCAAGACCTGCCAGGAC-3'</td>
<td>5'-GTAGTTAGTGAGCACAGGAC-3'</td>
<td>56</td>
</tr>
<tr>
<td>Flk-1</td>
<td>5'-CACCCTGGCCTCCTCAACCTCC-3'</td>
<td>5'-GATTTCATCCCACTACCAAAAGG-3'</td>
<td>56</td>
</tr>
<tr>
<td>Fhl-4</td>
<td>5'-AGGGCCATAGACCGAGAAGG-3'</td>
<td>5'-CTCTGCCAGATTCTTATTC-3'</td>
<td>56</td>
</tr>
<tr>
<td>c-fms</td>
<td>5'-TGGAAGTCTGCACTTCGGC-3'</td>
<td>5'-GCTGACTGTGATGCTGTC-3'</td>
<td>56</td>
</tr>
<tr>
<td>P.U.1</td>
<td>5'-GGCACTGAGCGACACAGAAGCT-3'</td>
<td>5'-GCTGACAGCTTTTGTAGGAGAC-3'</td>
<td>56</td>
</tr>
<tr>
<td>Ikars</td>
<td>5'-AGGAGCTTAAAGGAGACTCG-3'</td>
<td>5'-GTAGCTGACTTTTGTAGGAGAC-3'</td>
<td>56</td>
</tr>
<tr>
<td>Il-β</td>
<td>5'-CATGGAGGACTGAGGAGCCAC-3'</td>
<td>5'-CTCTGAGACCTACTTACAC-3'</td>
<td>54</td>
</tr>
<tr>
<td>Il-3</td>
<td>5'-TCAGACTTATTGTTCTCCGT-3'</td>
<td>5'-GCTGAGAAGGCGAAGGAG-3'</td>
<td>50</td>
</tr>
<tr>
<td>Il-4</td>
<td>5'-ACGCCAGATGATGTGGCCAAACCTC-3'</td>
<td>5'-GATGGAATCCATTTTGCTGATGAG-3'</td>
<td>56</td>
</tr>
<tr>
<td>Il-6</td>
<td>5'-GATGACATCTTCAGGACAGGATATC-3'</td>
<td>5'-TACCTCAGTTGACTTAAGTCC-3'</td>
<td>53</td>
</tr>
<tr>
<td>M-CSF</td>
<td>5'-GACAGGCGCTGAGCAGGGGAGGAC-3'</td>
<td>5'-ATAGAATGTTAAGTGAGACTCAG-3'</td>
<td>56</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5'-ATCCGAGTTAGGCTGCAAGAGCACC-3'</td>
<td>5'-ATTCTAGGAGGACTTGTGCAACAGG-3'</td>
<td>54</td>
</tr>
<tr>
<td>SCF</td>
<td>5'-TCAGGAGCAGGAGGAGCTC-3'</td>
<td>5'-TCAGGAGCAGGAGGAGCTC-3'</td>
<td>56</td>
</tr>
</tbody>
</table>

Primary TECs and establishment of ORTEC

E19 fetal thymus were disaggregated by treatment with collagenase and trypsin and filtered through a 40-μm mesh. The recovered fetal thymic macrophages with CD4 were suspended in the culture medium composed of RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, and gentamycin sulfate, and were inoculated onto six-well culture plates (4 × 10^6 cells/well) in the presence of 10 ng/ml OSM (R&D Systems, Minneapolis, MN). After a few days, floating cells were removed by washing with PBS. Adherent cells were analyzed after 1 wk of culture. For coculture experiments, primary TECs cultured for 10–14 days were used. To establish the cell line, ORTEC, primary TECs were maintained for 2 mo in the presence of OSM, and growing cells were cloned by a cloning cup.

Culture of fetal thymocytes

Thymocytes isolated from E14.5 fetal thymus were cultured under various conditions. In coculture experiments, thymocytes were cultured with semi-confluent ORTEC in the presence of OSM, stem cell factor (SCF; provided by Kirin, Takasaki, Japan) and IL-7 (PeproTech, Rocky Hill, NJ). The culture medium used was RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME and gentamycin sulfate (the standard culture medium). After 4 days, both floating and adherent cell numbers were determined, and the surface phenotype of these cells was analyzed by flow cytometry. Adherent cells were harvested by trypsin treatment and were filtered through a 40-μm mesh. Both floating and adherent cells were analyzed by flow cytometry. To test the requirement of cell-cell contacts, intra-TECS cultured for 10–14 days were used. To establish the cell line, ORTEC, primary TECs were maintained for 2 mo in the presence of OSM, and growing cells were cloned by a cloning cup.

Preparation of progenitor cells

Cell sorting was performed by FACSVantage SE (BD Biosciences). For the isolation of CD25^+/CD44^+/FcγRII^- cells, thymocytes without pretreatment with blocking Ab were incubated with FITC-Flk-1, PE-CD25, and biotin-CD44. Hemopoietic stem cells were isolated basically according to previous reports (12, 27, 28). Briefly, bone marrow and fetal liver cells were incubated with unconjugated rat Abs specific for lineage (Lin) markers (CD4, CD8, CD11b, Gr-1, B220, TER119). Lin^- cells were partially removed with sheep anti-rat IgG-conjugated immunomagnetic beads. Remaining cells were stained with FITC-Scal and PE-c-Kit Abs. Scal/c-Kit DP cells were used as hemopoietic stem cells fractions.

RT-PCR

RNA was prepared by using the Fast track kit (Invitrogen Life Technologies, Carlsbad, CA) or the RNeasy kit (Qiagen, Valencia, CA). According to the manufacturer’s protocol, RNA was reverse-transcribed using the first strand cDNA synthesis kit (Pharmacia, Peapack, NJ). PCR was performed using various sets of primers listed in Table 1. After incubation for 5 min at 94°C, PCR amplification was performed using the GeneAmp PCR system (PerkinElmer, Wellesley, MA) under the following conditions: denaturation at 94°C for 30 s; annealing at 54°C, 56°C, or 60°C for 30 s;
elungation at 72°C for 45 s. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide. GAPDH was used as an internal control.

**Phagocytosis assay**

CD4\(^+\)CD11b\(^+\) cells and CD4\(^-\)/CD11b\(^-\) cells were isolated from E14.5 thymus by FACSVantage. Cocultured cells were sorted by CD4 expression with FACSVantage. The cells were plated in parafilm chamber slides (Nalge Nunc) and incubated at 37°C for 2 h. For induction of apoptosis, thymocytes were incubated at 37°C for 12 h with 10\(^{-7}\) M dexamethasone in the standard culture medium. Apoptosis 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 assay. Apoptotic thymocytes (4\(\times\)10\(^6\) cells) were added to macrophages on chamber slides after fixation, macrophages were extensively washed three times. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, followed by three washes with PBS. After fixation, macrophages were stained with PE-conjugated CD11b. Phagocytosis was evaluated by microscopic observation.

**Immunostaining of cultured cells**

The Abs used were: anti-keratin 5 (Babco, Richmond, CA), biotin-UEA1, and isotype-matched Abs as negative controls for the first Abs. Troma-1, rat anti-keratin 8 mAb, was kindly provided by Dr. R. Kemler (Max-Planck Institute of Immunobiology, Freiburg, Germany). Cultured cells were fixed with 4% paraformaldehyde in PBS and stained with Abs; incubated with HRP-conjugated anti-rat, anti-rabbit IgG, or HRP-conjugated streptavidin; and visualized by diaminobenzidine.

**Results**

**ORTEC**

As it was recently reported that E14.5 thymocytes give rise to CD11b\(^+\) cells in the presence of M-CSF, IL-6, and IL-7 (14), we cultured E14.5 thymocytes in the presence of these cytokines to test whether thymocytes give rise to CD4\(^+\) macrophages. Although CD11b\(^+\) cells were generated by these cytokines as reported previously, they never expressed CD4 (Fig. 1). Therefore, we suspected that the development of CD4\(^+\) macrophages might be supported by the thymic environment, but not soluble factors alone. OSM is abundantly expressed in the thymus (Fig. 2A) and OSMR is expressed at the periphery of medulla but not in the thymocytes (21), suggesting that OSM may play a role for thymic environment. As it is believed that thymic selection occurs in this area, we considered the possibility that TECs at the periphery of medulla might play a role for the development of CD4\(^+\) macrophages.

We first cultured TECs in the presence or absence of OSM. Whereas fibroblastic-like cells dominated the culture in the absence of OSM, the addition of OSM induced the outgrowth of epithelial-like cells (Fig. 2B). By maintaining the thymic epithelial culture in the presence of OSM, we were able to establish a cell line, ORTEC, that proliferated in response to OSM (Fig. 2B). ORTEC exhibited morphology similar to those primary TECs grown in the presence of OSM (Fig. 2B).

To characterize ORTEC, flow cytometric analysis was performed. As expected, they expressed OSMR\(\beta\) as well as gp130, the common receptor subunit of the IL-6 family cytokines (Fig. 3A). ORTEC did not express mature endothelial markers such as tyrosine kinase with Ig and epidermal growth factor homology domains (TIE)-1, TIE-2, and PECAM, suggesting that ORTEC cells are not endothelial cells (Fig. 3A). To further characterize ORTEC, we used macroarray analysis using 1000 known genes, and found that ORTEC expressed various epithelial genes such as cadherin-6, cadherin-11, cadherin-13, and \(\beta\)-catenin. ORTEC also expressed CD29, CD49C, CD104, and connexin-43 (data not shown), which were reported to be expressed in TECs (29). MHC class II was also expressed in ORTEC at a low level (data not shown). It is known that TECs in cortex and medulla can be distinguished by the expression of keratins, i.e., keratin 5 is expressed in medulla, whereas keratin 8 is expressed in cortex (15). Immunostaining indicated that ORTEC expressed keratin 5 but not keratin 8 (Fig. 3B). In addition, expression of UEA-1, a marker for the

**FIGURE 1.** Soluble factors fail to induce CD4\(^+\) macrophages. E14.5 thymocytes were cultured in the presence of a mixture of cytokines (M-CSF, IL-6, OSC, SCF, and IL-7) for 4 days. A, Expression of CD4 and CD11b was examined by flow cytometry. B, The percentage of CD4\(^+\)/CD11b\(^+\) cells in the total CD11b\(^+\) cells. CD4\(^-/\)CD11b\(^+\) cells were not generated from E14.5 thymocytes by the cytokine mixture.

**FIGURE 2.** Establishment of TEC line, ORTEC. A, RT-PCR analysis of OSM. RNA was prepared from thymus at various developmental stages and used for RT-PCR analysis. B, Cells from E19 thymus were cultured in the absence (a) or presence (b) of OSM. In the presence of OSM, many epithelial/endothelial cell-like clusters appeared. The ORTEC was established by maintaining the culture for more than 2 mo. Morphology of ORTEC in the absence(c) or the presence (d) of OSM. Original magnification was \(\times20\).
thymic core medullary epithelium (30), was not detected in ORTEC (Fig. 3D). These results suggest that ORTEC represent epithelial cells at the periphery of medullar.

**Generation of CD4+ macrophages from thymocytes by ORTEC**

We performed coculture experiments of immature thymocytes with TECs. We cocultured E14.5 thymocytes in the presence of SCF, IL-7, and OSM with primary TECs that had been grown in the presence or absence of OSM. CD4+/CD11b+ cells vigorously proliferated in the presence of primary TECs that had been grown in the presence of OSM. In contrast, production of CD4+/CD11b+ cells was less effective in the coculture with primary TECs that had been grown without OSM (Fig. 4A). To uncover the role of OSM in the development of CD4+ macrophages in vivo, we analyzed gp130<sup>+/+</sup> thymus. OSM manifests its functions through the receptor consisting of OSMRβ and gp130. As gp130<sup>−/−</sup> mutant lacks its entire cytoplasmic domain, the mutant mice fail to respond to OSM. The population of CD4+/CD11b+ cells in gp130<sup>−/−</sup> thymus was smaller than that of the wild-type littermates (Fig. 4B, upper panel), although it was reported that the development of T cell progenitors was not affected in the gp130<sup>−/−</sup> mice (31). The ratio of CD4+/CD11b+ cells to the total CD11b+ cells was significantly reduced in gp130<sup>−/−</sup> thymus (Fig. 4B, lower panel). This supports the possibility that OSM plays a role for the development of CD4+/CD11b+ cells.

To analyze the induction of CD4+ macrophages from thymocytes in more detail, we used the TEC cell line, ORTEC, for coculture experiments. Coculture of thymocytes with ORTEC also resulted in the production of CD4+/CD11b+ cells (Fig. 5A). After 4 days of coculture, both floating and adherent cells were increased (Fig. 5B). Flow cytometric analysis of the floating cells revealed that almost all thymocytes became CD4+/CD11b+ (Fig. 5A). In sharp contrast, almost all cocultured adherent cells were CD4−/CD11b+ (Fig. 5A, note that CD11b− cells in adherent cells were ORTEC).

To further characterize cocultured CD4+/CD11b+ cells, FACS analysis was performed. MHC class II, CD80, and CD86 were expressed on these cocultured cells (Fig. 6A). The expression profile of these cocultured cells was CD4+/CD11b+<sup>+</sup>/CD11c<sup>−</sup>/MHCII<sup>+</sup>/CD80<sup>−</sup>/CD86<sup>+</sup>. This expression profile of cocultured cells is the same as that of freshly isolated CD4+ macrophages in the thymus (24). These results indicate that immature thymocytes differentiate to CD4+ macrophages in the presence of ORTEC. To confirm whether these CD4+ cocultured cells are scavengers for apoptotic thymocytes, we compared phagocytic activity of CD4−/CD11b+ cells produced in coculture with that of freshly isolated CD4+ macrophages from thymus (Fig. 6B). The CD4+/CD11b+ cells generated from thymocytes in vitro phagocytosed apoptotic thymocytes as strongly as freshly isolated CD4+ macrophages from thymus (Fig. 6B). These results indicate that CD4+ macrophages are derived in vitro from immature thymocytes in the presence of TECs.

**Cytokine requirement for the development of CD4+/CD11b+ cells**

Conditioned medium of ORTEC failed to induce CD4+/CD11b+ cells from thymocytes, whereas it induced the development of
These data suggest that CD4+CD11b−/H11001 intrathymic lymphoid progenitors. Cells produced by coculture from thymocytes. Error bars indicate SD. Ab is shown as negative control. (Fig. 8). In the absence of SCF and IL-7, CD4+ cells were not induced by the addition of SCF and IL-7 significantly augmented by the addition of SCF and IL-7 (Fig. 8 B). Numbers shown are fold expansion of cells produced by coculture from thymocytes. Error bars indicate SD. Ikaros in CD4+ thymocytes can be separated into two populations based on the expression of CD25 and CD44, and the CD25−CD44+ cells were generated from this population (E14.5 DN thymocytes). Flow cytometric analysis of surface Ags on cocultured CD4+CD11b+ cells (Fig. 7 A). Proliferation of immature thymocytes in the presence of ORTEC (Fig. 5). In the absence of SCF and IL-7 (Fig. 8), CD4+CD11b+ cells were not induced when the direct interaction between thymocytes and ORTEC was prevented by using a Transwell chamber (Fig. 7 A). RT-PCR analysis revealed that ORTEC expressed mRNA for IL-1β, IL-6, M-CSF, and SCF (Fig. 7 B), suggesting that CD4+ macrophages were not induced by these cytokines (see also Fig. 1). It is thus strongly suggested that a direct interaction between thymocytes and ORTEC is required for the development of CD4+ macrophages.

Proliferation of immature thymocytes in the presence of ORTEC was significantly augmented by the addition of SCF and IL-7 (Fig. 8 A). In the absence of SCF and IL-7, CD4+CD11b+ cells did not proliferate and were not induced by coculture with ORTEC (Fig. 8 A). In contrast, CD4+CD11b+ cells were induced in the absence of SCF and IL-7, although the induction efficiency was lower than that in the presence of all cytokines. These findings suggest that IL-7 is a crucial factor for the induction of CD4+CD11b+ cells in this coculture condition, although the requirement of SCF for the development of CD4+ macrophages from thymocytes is unclear as ORTEC constitutively expressed SCF (Fig. 7 B). Furthermore, semiquantitative RT-PCR analysis demonstrated that the CD4+CD11b+ cells expressed Ikaros, a transcription factor for lymphoid, but not for myeloid cells. The expression level of Ikaros in CD4+CD11b+ cells was comparable to that of E14.5 thymocytes (Fig. 8 B), indicating that Ikaros expression in CD4+CD11b+ cells was not due to the contamination of thymocytes. These data suggest that CD4+/CD11b− cells were generated from intrathymic lymphoid progenitors.

Intrathymic lymphoid progenitors give rise to CD4+ macrophages

E14.5 DN thymocytes can be separated into two populations based on the expression of CD25 and CD44, and the CD25−CD44− thymocytes are the most immature T cell progenitors in the thymus. While it was also reported that CD25−CD44+ cells gave rise to macrophages in the presence of M-CSF, IL-6, and IL-7 (14), the CD25−CD44+ cells in fetal thymus were heterogeneous and contained both lymphoid and myeloid precursors (32). It was shown that the FcR+ subpopulation of the CD25−CD44+ cells is comprised of almost exclusively T cell progenitors and contamination of myeloid progenitors is minimum (32). Therefore, we isolated the CD25−CD44+/FcR− population from E14.5 thymus and examined their differentiation in the presence of ORTEC. As shown in Fig. 9, CD4+CD11b+ cells were generated from this population by coculture with ORTEC. While CD11b+ cells were developed from CD25−CD44+/FcR− thymocytes by a mixture of cytokines—OSM, SCF, M-CSF, IL-6, and IL-7—they were negative for CD4, highlighting the difference between CD4−CD11b+ cells and CD4+CD11b+ cells. In addition, the generation of CD4+/CD11b− cells was completely dependent on IL-7 (Fig. 9), an essential cytokine for T cell development by maintaining survival of T cell precursors (19, 33). In the absence of IL-7, almost all CD25−CD44+/FcR− cells died during 4 days of culture, while only a small number of CD11b+ cells were found. These results indicate that CD4+/CD11b− cells are generated directly from CD25−CD44+/FcR− thymocytes, but not as a result of expansion of a small population of CD11b+ cells. Collectively, the IL-7 dependency also strongly supports that CD4+/CD11b− cells are generated from the intrathymic lymphoid progenitors.

We also examined whether multipotent or myeloid-restricted progenitors might generate CD4+ macrophages (Fig. 10). Hemopoietic stem cells (HSCs) and the monocytes/macrophage (Mo/Md) fraction were isolated and cocultured with ORTEC. HSCs from fetal bone marrow did not proliferate and differentiate into CD4+CD11b+ cells (Fig. 10, upper panel). HSCs from fetal bone marrow. CD45+ cells were generated from bone marrow did not proliferate and differentiate into CD4+CD11b+ cells (Fig. 9). Co-cultured CD4+CD11b+ cells were identified as macrophages (Fig. 6 B) by cytometric analysis of surface Ags on macrophages. Error bars indicate SD. Note that thymic macrophages used in this experiment are CD11b+ cells in the thymus.

**FIGURE 5.** Development of CD4+/CD11b− cells by ORTEC. A. Thymocytes from E14.5 embryos were seeded onto ORTEC in the presence of SCF, IL-7, and OSM. After 4 days of coculture, floating cells and adherent cells were recovered. The expression of CD45, CD11b, and CD4 was analyzed by FACS. CD45− cells are ORTEC. Staining of isotype-matched Ab is shown as negative control. B. Numbers shown are fold expansion of cells produced by coculture from thymocytes. Error bars indicate SD.

**FIGURE 6.** Characterization of CD4+/CD11b+ cells produced by coculture. A. Flow cytometric analysis of surface Ags on cocultured CD4+/CD11b+ cells. E14.5 thymocytes were cocultured with ORTEC for 4 days, and the expression profile of surface markers for dendritic cells was examined. B. Phagocytic assay. Thymocytes were treated with dexamethasone and apoptotic thymocytes were stained with DAPI. Those apoptotic cells were then incubated with macrophages. The efficiency of phagocytosis was evaluated by counting apoptotic nuclei incorporated in the macrophages. The numbers of DAPI-positive cells per macrophage are shown. Error bars indicate SD. Note that thymic macrophages used in this experiment are CD11b+ cells in the thymus.
liver vigorously proliferated, and CD4+/CD11b+ cells were induced by this coculture. Although a small number of CD4+/CD11b+ cells were detected in the coculture of fetal liver HSCs, the induction efficiency of CD4+/CD11b+ cells was much lower than that from intrathymic lymphoid progenitors (Fig. 10, lower panel). Taken together, it is suggested that thymic CD4+ macrophages are not derived from the myeloid and multipotent progenitors.

Discussion

Macrophages are present in various tissues and develop from myeloid progenitors. Macrophages are differentiated from bone marrow cells in vitro by cytokines such as M-CSF and GM-CSF. However, the CD4+ macrophages described in this paper are derived from the most immature lymphoid progenitors, CD25+/CD44+/FcR+ cells in the thymus. This situation is analogous to the development of DCs. Two distinct classes of DCs are known: one is generated from myeloid progenitors that also give rise to macrophages and the second, thymic DC, is derived from lymphoid progenitors. In myeloid progenitors that give rise to macrophages and DCs, the direction of differentiation is regulated by cytokines. We found that immature thymocytes differentiated to CD4+ macrophages by ORTEC as well as primary TECs grown in the presence of OSM (Fig. 4A). Interestingly, although conditioned medium of ORTEC induced development of CD4+/CD11b+ cells, it failed to induce the development of CD4+ macrophages, suggesting that the development of CD4+ macrophages from the lymphoid progenitors requires a direct contact with TEC.

M-CSF is known as a primary regulator of macrophage production. However, Cecchini et al. (34) reported that macrophages were present in the thymus of op/op mouse, which lacks M-CSF due to an inactivating mutation in the M-CSF gene. Lee et al. (14) reported that macrophages were not induced from thymocytes by M-CSF alone, and the combination of M-CSF/IL-6 induced significant induction of macrophages. Consistent with their results, some CD4+/CD11b+ cells were generated from CD25+/CD44+/FcR+ thymocytes in the presence of M-CSF and IL-6 (Fig. 9). We also demonstrated that the population of CD4+/CD11b+ cells was not reduced by the lack of gp130 signals (Fig. 4B), suggesting that the IL-6 family of cytokines is not essential for the development of CD4+ macrophages in the thymus. However, a mixture of cytokines as well as conditioned medium of ORTEC, both of which contained M-CSF and IL-6, failed to induce CD4+/CD11b+ cells, highlighting a difference between CD4+/CD11b+ cells and CD4+/CD11b+ cells. In contrast to the development of CD4+/CD11b+ cells that is induced by soluble factors, development of CD4+ macrophages requires the cell-cell contacts between thymocytes and ORTEC. To understand the mechanism underlying the development of CD4+ macrophage, it is necessary to identify the molecules responsible for the conversion of thymocytes to macrophages. Attempts to find such molecules are currently underway.

Our results indicate that CD4+ macrophages are derived from the most immature thymocytes with the CD25+/CD44+/FcR+ phenotype in an IL-7-dependent manner. The frequency of macrophages generated from CD25+/CD44+/FcR+ thymocytes was dramatically increased by the addition of IL-7, a cytokine that delivers survival signals for lymphoid cells (33, 35). Although CD25+/CD44+/FcR+ thymocytes may be still heterogeneous (32), IL-7 dependency of the CD4+ macrophage development strongly suggests lymphoid progenitors as the origin of such macrophages in the thymus. Myeloid (Mo/Md) progenitors in bone marrow failed to differentiate into CD4+ macrophages by coculture with ORTEC (Fig. 10). In contrast, a small number of CD4+/CD11b+ cells were generated from fetal liver HSCs. As the HSC fraction from fetal liver includes lymphoid- as well as myeloid-restricted progenitors and multipotent stem cells are not present in fetal thymus (36), it is likely that lymphoid-restricted progenitors in the HSC fraction differentiated into CD4+ macrophages by coculture with ORTEC. Taken together, it is concluded that CD4+ macrophages are derived from the most immature lymphoid progenitors.
macrophages are derived from intrathymic lymphoid progenitors but not from other progenitors.

Although heterogeneity of TECs has been known, little is known about the characteristics of TECs. In fact, only a few Ags have been known to distinguish between cortex and medullar epithelial cells. Hence it has been difficult to study the characteristics of each subset of TECs. We found that OSM stimulates the proliferation of TECs in the primary culture of fetal thymic cells and established the OSM-dependent cell line ORTEC that expressed various epithelial markers together with OSMR. A cell population that expressed OSMR was readily found in thymic cells by flow cytometry (data not shown). While these sorted OSMR+ cells survived, they did not proliferate in the presence of OSM alone, suggesting that they require additional factors that are presumably provided by cells in the primary culture. As OSMR was not expressed on thymocytes and CD4+CD11b+ cells, the effect of OSM on the development of these cells was considered to be indirect (Fig. 8B). Therefore, it is most likely that OSM regulates the development of CD4+ macrophages through the thymic environment. Consistently, ORTEC, as well as primary TECs cultured in the presence of OSM supported the development of CD4+ macrophages from immature thymocytes. Moreover, in gp130(Delta) thymus, the population of CD4+/CD11b+ cells was significantly reduced compared with CD4+/CD11b+ cells (Fig. 4B) and our preliminary results using OSM-deficient mice also show similar phenotype (data not shown). The results strongly suggest that OSMR+ TECs play an important role for the differentiation of thymocytes by modulating the generation of CD4+ macrophages in the thymus. A conditional knockout mouse of the STAT3 gene using the CRE recombinase driven by the keratin 5 promoter exhibited severe thymic hypoplasia and alterations in the cortical epithelial cell architecture. Moreover, many apoptotic thymocytes were found in the thymus of knockout mice (37). Keratin 5 is expressed in the majority of TECs in the medulla and corticomedullary junction and is also expressed in ORTEC. STAT3 is a major mediator of OSM signals and defective OSM signaling in the conditional knockout mice may lead to hypoplasia of TECs, which impairs the development of CD4+ macrophages and thymic DCs. These results strongly support our hypothesis that the normal TECs are involved in the development of DC and CD4+ macro-

![FIGURE 9. Generation of CD4+ macrophages from FcR+/CD44+ /CD25+ thymocytes. FcR+/CD44+ /CD25+ cells were sorted from E14.5 thymocytes, and 2 x 10^6 cells were cultured for 4 days in a six-well plate. Cells were recovered and cell numbers were determined. The ratio of each population was determined by FACS. Error bars indicate SD.](http://www.jimmunol.org/)

![FIGURE 10. Development of CD4+ macrophages from HSCs. HSCs (c-Kit+/Sca1-/Lin-) were recovered from bone marrow (BM) or fetal liver (FL) as described in Materials and Methods. CD11b+ cells from bone marrow were used as the Mo/Mb fraction. Isolated progenitor cells were cocultured with ORTEC for 4 days in the presence of SCF, IL-7, and OSM. Cocultured cells were recovered and cell numbers were determined. The ratio of each population was determined by FACS. The fold expansion shown is relative to the numbers of input cells to the culture. Error bars indicate SD.](http://www.jimmunol.org/)