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*J Immunol* 2001; 167:2511-2521; doi: 10.4049/jimmunol.167.5.2511

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Expression of $\alpha_4\beta_7$ Integrin Defines a Distinct Pathway of Lymphoid Progenitors Committed to T Cells, Fetal Intestinal Lymphotoxin Producer, NK, and Dendritic Cells

Hisahiro Yoshida,‡ Hiroshi Kawamoto,† Sybil M. Santee,‡ Hiroyuki Hashi,* Kenya Honda,* Satomi Nishikawa,* Carl F. Ware,‡ Yoshimoto Katsura,‡ and Shin-Ichi Nishikawa*

During embryogenesis, the Peyer’s patch anlagen are induced by a cell population that produces lymphotoxin (LT) $\alpha_4\beta_7$, following stimulation of IL-7R$\alpha$. In this study, we show that the LT-producing cell is localized within the IL-7R$\alpha^+$ and integrin $\alpha_4\beta_7$ ($\alpha_4\beta_7^+$) population in the embryonic intestine. Lineage commitment to the LT producer phenotype in the fetal liver coincides with expression of $\alpha_4\beta_7$. Before expression of $\alpha_4\beta_7$, the potential of IL-7R$\alpha^+$ population to generate B cells is lost. However, the progenitors for T cells and LT producer cells reside in the IL-7R$\alpha^+\alpha_4\beta_7^+$ cells, but during subsequent differentiation, the potential to give rise to T cells is lost. This IL-7R$\alpha^+\alpha_4\beta_7^+$ population migrates to the intestine, where it induces the Peyer’s patch anlagen. When stimulated with IL-15 or IL-3 and TNF, the intestinal IL-7R$\alpha^+\alpha_4\beta_7^+$ population can differentiate into fully competent NK1.1$^+$ NK cells or CD11c$^+$ APCs. Expression of $\alpha_4\beta_7$ is lost during differentiation of both lineages; IL-7R$\alpha$ expression is lost during NK1.1$^+$ cells differentiation. A newly discovered lineage IL-7R$\alpha^+\text{c-KIT}^+\alpha_4\beta_7^+$ population in the fetal liver is committed to T, NK, dendritic, and fetal intestinal LT producer lineage, the latter being an intermediate stage during differentiation of NK and dendritic cells. The Journal of Immunology, 2001, 167: 2511–2521.

Although these two signal transduction pathways are independent of each other per se, we recently identified a cell population that serves as the convergence point of the two signals (10). This population bears IL-7R$\alpha$, CD45, c-KIT, CD44, but none of the lineage markers such as CD3, B220, CD19, NK1.1, CD11b, CD11c, Gr-1, or ter119 (Lin). This surface phenotype is reminiscent of lymphoid progenitors (11), indicating a close relationship of this population to lymphocytes. A fraction of this population expresses LT$\alpha_4\beta_7$ and is suppressed in vivo by treatment with an antagonistic anti-IL-7R$\alpha$ mAb (10). This strongly suggests that activation of the IL-7R$\alpha^+\text{c-KIT}^+\alpha_4\beta_7^+$ pathway of these cells leads to the induction of LT$\alpha_4\beta_7$, which subsequently activates surrounding LT$\beta R^+$ cells to form VCAM-1$^+\text{ICAM-1}^+$ PP anlagen (10). We refer to this Lin$^+\text{IL-7R}^+\alpha_4\beta_7^+$ population as fetal intestinal LT producer (FILyP) cells, as it is the only cell type in the fetal intestine to express LT$\alpha_4\beta_7$ (10). Differentiation of IL-7R$\alpha^+\text{CD3}^+\text{CD4}^+$ cells is inhibited in mice with the null mutation of the Id2 gene (12). PP anlagen are not induced in these mice, indicating unequivocally that generation of IL-7R$\alpha^+\text{CD3}^+\text{CD4}^+$ cells in the embryonic intestine is indeed essential for the induction. Based upon these observations, we have proposed that Lin$^+\text{IL-7R}^+\alpha_4\beta_7^+$ subpopulation in the embryonic intestine includes the inducer cells of PP anlagen (10).

As this surface phenotype is common to several lymphoid progenitors, such as common lymphoid progenitors, T/NK/dendritic cell (DC)-committed progenitors, and B-committed progenitors (11, 13–16), we investigated the relationship between FILyP cells and other lymphoid cell lineages. Our results show that commitment to FILyP cells is characterized by the expression of $\alpha_4\beta_7$ integrin ($\alpha_4\beta_7$), an adhesion molecule that plays important roles in the migration of CD4$^+\text{CD3}^+\text{LT}^+$ cells to the neonatal LN (17). Upon commitment, the $\alpha_4\beta_7^+$ populations can give rise to T and FILyP cells, but not B cells. This population subsequently loses the ability to produce T cells (T-potential) and then migrates to the intestine. Our results further demonstrate that the IL-7R$\alpha^+\alpha_4\beta_7^+$...
population in which FILyP cells are included represents an intermediate progenitor of NK and DC.

Materials and Methods

Mice

Pregnant C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). Ly-5.1+ C57BL/6J mice were maintained in our animal facility, as described (18). Female and male mice were mated overnight, and those with a vaginal plug were judged pregnant. Noon of the day when the vaginal plug was identified was calculated as 0.5 dpc.

Abs and rLTβR human IgG1 chimerical protein

Names of clones and sources of mAbs used in this study are listed below: Anti-IL-7Ra Ab (A7R34) and anti-ε-Kit Ab (ACK2) were established in our laboratory and prepared, as previously described (15, 19). Anti-B220 Ab (RA3-6B2), anti-CD11b Ab (Mac-1), anti-CD8α (53-6.7), and anti-F4/80 (F4/80) were purified from hybridoma culture supernatant, as described (14). Anti-Thy-1.2 was purchased from Caltag Laboratories (San Francisco, CA); anti-CD3 (Y65.135) was purchased from Seikagaku-kogyo (Tokyo, Japan); anti-integrin αβ, heterodimer (DATK32), anti-CD3 (2C11), anti-CD4 (GK1.5), anti-CD11c (HL3), anti-TCR-β (H57-597), anti-TCR-γ (GL3), anti-CD45 (30F11.1), anti-NK1.1 (NKR-P1C), anti-Gr-1 (RB6-8C5), anti-CD44 (IM7), anti-CD25 (7D4), anti-CD80 (IG10), anti-CD86 (GL1), anti-Ly-49D (4E5), anti-2B4 (2B4), and anti-DX5 (DX5) were purchased from BD PharMingen (San Diego, CA).

The extracellular domain of the LTβR was fused to the human IgG1 Fc portion (20) and expressed in baculovirus (Invitrogen, Groningen, The Netherlands), and the chimeric protein was purified as described (21). This chimeric protein was used in flow cytometric analysis to detect membrane-anchored LTαβ, with biotinylated goat anti-human IgG (Southern Bio-technology Associates, Birmingham, AL) as the secondary reagent, followed by streptavidin conjugated with allophycocyanin (Molecular Probes, Eugene, OR). For a negative control, we used a fusion protein composed of the extracellular domain of platelet-derived growth factor receptor α and human IgG1 Fc domain (22).

Preparation of single cell suspension for flow cytometry and cell sorting

All organs of embryos were dissected with fine forceps under the stereomicroscope, and then dissociated with dispase (Life Technologies). In each PCR for detecting the concentration of RT-PCR products with HPRT primer sets at various concentrations, we have previously demonstrated that CD45+CD44+IL-7Rα+ε-Kit+αβ2+ Thy-1+/-CD4+/- cells in the embryonic intestine, which are also negative for the lineage markers B220, CD19, CD3, Ter119, Mac1, Gr1, CD11c, and NK1.1 (Lin), express LTα and LTβ mRNA (10). For convenience, we will call this population simply Lin-IL-7Rα+αβ2+ cell or FILyP cell. As induction of PP anlagen is dependent on LTαβ, PP inducers should be a component of this population.

Results

Maintenance and stimulation of the FILyP cell in culture

We have previously demonstrated that CD45+CD44+IL-7Rα+c-Kit+αβ2+ Thyl-1+/-CD4+/- cells in the embryonic intestine, which are also negative for the lineage markers B220, CD19, CD3, Ter119, Mac1, Gr1, CD11c, and NK1.1 (Lin), express LTα and LTβ mRNA (10). For convenience, we will call this population simply Lin-IL-7Rα+αβ2+ cell or FILyP cell. As induction of PP anlagen is dependent on LTαβ, PP inducers should be a component of this population.

First, we wanted to determine whether LTαβ production in this population is a direct consequence of the stimulation of IL-7Rα. To identify appropriate culture conditions for this population, we cultured Lin-IL-7Rα+αβ2+ cells in 15.5-dpc embryonic intestines with various cytokine combinations. A combination of SCF and IL-7 induced more than a 10-fold increase of Lin-IL-7Rα+αβ2+ cells in 5 days of culture (data not shown). As expected, virtually all the cells, both CD4+ and CD4−, in this culture expressed LTαβ (Fig. 1, A and B). A combination of IL-6 and SCF induced only a 2-fold increase in cell number (data not shown), and most cells in this culture did not express surface LTαβ (Fig. 1B). RT-PCR analysis of the same population demonstrated that LTα gene expression was almost absent, although low level LTβ gene expression could be detected (Fig. 1D, lane 0). Although mTBR-Fc, which is used to detect surface LTαβ, will also detect LIGHT (the cytokine that is homologous to lympho-toxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes), mHVEM-Fc (mouse herpes virus entry mediator soluble receptor), which binds LIGHT (30), did not bind FILyP cells ex vivo from 15.5-dpc embryonic gut nor those maintained in vitro, indicating the presence of LTαβ only (data not shown).

Cell culture

In some experiments, sorted cells were cultured on stromal cell layers. Preparation of TsT4 and Op9 stromal cell layers was as described previously (10, 23). Sorted cells were suspended in RPMI 1640 (Life Technologies) containing 10% FCS and 5 × 10−5 M 2-ME, and placed on the stromal cell layer with or without additional cytokines. These stromal cell lines have been shown to support differentiation of most blood cell lineages, including B lymphocytes from multipotent stem cells, although it has been difficult to induce mature T cells (23).

Unless indicated, U-bottom-shaped 96-well cluster dishes (Falcon) were used for all culture experiments without stromal cells. A total of 3000–5000 cells was cultured in each well. The concentrations of recombinant cytokines used in this study were murine stem cell factor (SCF; 100 ng/ml), murine IL-7 (20 U/ml), human IL-6 (20 U/ml) (Life Technologies), simian IL-15 (20 ng/ml) (Genzyme, Cambridge, MA), murine IL-3 (20 U/ml), murine GM-CSF (20 ng/ml), and murine TNF-α (20 ng/ml) (PeproTech, Rocky Hill, NJ).

In vitro assays

Colony-forming cell assay in the semisolid medium containing methylcellulose was performed as described previously (24). In this study, we used GM-CSF (200 ng/ml; R&D Systems, Minneapolis, MN) or murine M-CSF (100 ng/ml; R&D Systems).

The T-potential was assessed by using a high oxygen thymus organ culture system, as described previously (25). A total of 3–100 cells was placed in wells containing a deoxyguanosine (Sigma)-treated thymic lobe. The cultures were maintained in the presence of 100 ng/ml SCF, 10 U/ml IL-7, and 30 U/ml IL-3. To facilitate the colonization of the inoculated cells, thymic lobes were cut into four pieces, which sealed spontaneously to form a lobe during incubation (26). Five cultures were prepared for each assay. After incubating for 14 days, each lobe was dissociated into single cell suspensions, split to aliquots, and three-color stained with mAb mixtures. Only cells with the light scatter profile of lymphocyte were gated and analyzed.

The NK activity was assessed by using YAC-1 target cells. In this experiment, we used the fluorescent dye release assay (27, 28). DC activity was assessed by [3H]Thymidine incorporation of CD3+CD4+ T cells from BALB/c mice, according to the method described by Bjorck et al. (29).

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Using this LTαβ-negative population, we investigated whether LTαβ expression can be induced by stimulation of IL-7Rα. As clearly seen in Fig. 1C, LTαβ expression became detectable as early as 1 h after addition of IL-7 and reached a peak after 3 h. This prompt induction of LTαβ expression was also confirmed by RT-PCR analyses of LTα and LTβ mRNA (Fig. 1D, lanes 1 and 3). LTαβ protein expression level was stable even 24 h after IL-7 stimulation, but the mRNA transcription level seemed gradually down-regulated at that time (data not shown). These results indicate that LTαβ production in this population is a direct consequence of IL-7Rα stimulation. It should be noted that virtually all Lin−IL-7Rα+αβ+ cells responded to IL-7 by expressing LTαβ, implying that most Lin−IL-7Rα+αβ+ cells have the potential to be involved in PP induction.

To confirm that LTαβ expression of Lin−IL-7Rα+αβ+ cells is not the artifact induced in these in vitro culture conditions, we examined LTαβ expression of freshly isolated Lin−IL-7Rα+αβ+ cells from 16.5-dpc mouse intestine. Similar to our previous analyses of mRNA expression (10), we found both CD4+ and CD4−Lin−IL-7Rα+αβ+ cell population expressed LTαβ on their surface (Fig. 1, E and F). In contrast to the in vitro culture, however, only a subset of the Lin−IL-7Rα+αβ+ cells produced LTαβ in vivo.

Expression of αβ in the Lin−IL-7Rα+ population is an early step during divergence of the FILyP cell population from the conventional lymphocyte

Lin−IL-7Rα+αβ+ cells in the mesenteric regions are lymphoid in morphology (10, 17). However, this population in the intestine could not give rise to T nor B cells under culture conditions that support the differentiation of lymphocytes (data not shown), indicating that it has already diverged from conventional lymphocytes. We speculated that the divergence of this lineage from the conventional lymphocyte occurs in the hemopoietic organs, and therefore investigated the differentiation potential of fetal liver cells.

Lin−IL-7Rα−c-Kit+CD45+, Lin−IL-7Rα+αβ−, and Lin−IL-7Rα+αβ+ cells were purified from fetal livers of 12.5-dpc embryos and subjected to various progenitor assays (Fig. 2). When 100 cells from each population were cultured on the Tissue culture cell line in the presence of IL-7 and SCF, B cells were generated (B-potential) from Lin−IL-7Rα−c-Kit+CD45+, and Lin−IL-7Rα+αβ+ populations, but not from the Lin−IL-7Rα+αβ− population (Fig. 3, A and C). Even using 5000 of Lin−IL-7Rα+αβ− cells for cocultivation with stromal cells, we found no B-potential in that population (data not shown). In contrast, CD4+CD3− cells that have been implicated in induction of PP and LN (10, 17) were generated from both Lin−IL-7Rα+αβ+ and Lin−IL-7Rα−αβ− populations (Fig. 3, B and D). The CD4+CD3− cells were more frequently generated from Lin−IL-7Rα+αβ− population than from Lin−IL-7Rα+αβ− population. Because myeloid cell differentiation occurred in the culture of Lin−IL-7Rα+αβ− population at very low frequency (0.3% in Fig. 3A), we assessed the frequency of colony-forming cells reactive to either GM-CSF or M-CSF. The frequency of the granulocyte-macrophage CFU or macrophage CFU was 100-fold lower in Lin−IL-7Rα+αβ− population than Lin−IL-7Rα−c-Kit+CD45+ population, and nearly absent in Lin−IL-7Rα+αβ− population (Table 1).

We next assessed the potential of Lin−IL-7Rα+αβ− and Lin−IL-7Rα+αβ+ populations to give rise to T cells and FILyP cells. A total of 100, 30, 10, and 3 cells from each population was cultured in deoxyguanosine-treated thymic lobes from Ly-5.1 mice in the presence of IL-3, IL-7, and SCF (26). This system has been...
shown to allow differentiation of T cells from most immature pluripotent hematopoietic stem cells (18). Fourteen days after incubation under this high oxygen atmosphere, the lobes were harvested separately and analyzed for growth of Ly-5.2+ donor cells, αβ and γδ T cells, and CD4+CD3− Filyp cells. CD8+ cells were excluded to distinguish Filyp cells from CD8+CD4−CD3− immature T cells. Although the CD4− fraction can express LTα1β2 (10) (Fig. 1A), only CD4+CD3− cells were scored as the Filyp cells, as not all CD4+CD3− cells represent Filyp cells. Consequently, we may have underestimated the frequency of the progenitor of the Filyp cells in this experiment. Flow cytometric analyses of thymic lobes, which received three cells, are illustrated in Fig. 4, and all results are summarized in Table II. The potential to give rise to T cells and Filyp cells was observed both in αββ− and αββ+ fractions, but the αβ− fraction contained a higher frequency of T progenitors. Moreover, all lobes in which differentiation of CD3+ T cells were observed contained CD4+CD3− Filyp cells (Fig. 4).

The above data suggest that the differentiation of Filyp cells progresses from IL-7Rαc−Kit+αβ− to IL-7Rαc−Kit+αβ− and subsequently to IL-7Rαc−Kit+αβ− stages. During this course, myeloid and B cell potentials are lost sequentially, upon expression of IL-7Rα and αβ, respectively. Although some T cell potential appears to be retained after expression of αβ, it is eventually lost from the αββ− population in the fetal liver.

To confirm differentiation from IL-7Rααβ− to IL-7Rααβ− populations, we sorted Lin−IL-7Rααβ− cells from the liver of 15.5-dpc embryos and cultured them on OP9 stromal cell layer for 40 h in the presence of IL-7 and SCF. During this short-term culture, the cell number increased ~6-fold and

Table I. Potential of Lin−IL-7Rααβ− and Lin−IL-7Rααβ− cells to form M-CSF or GM-CSF induced colonies

<table>
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<tr>
<th></th>
<th>Lin−IL-7Rααβ−</th>
<th>Lin−IL-7Rααβ−</th>
<th>Lin−IL-7RαCD45+</th>
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<td>CFU-M</td>
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<td>0</td>
<td>106 ± 22</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>2.5 ± 1.7</td>
<td>0.25 ± 0.5</td>
<td>244 ± 40</td>
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</table>

* Lin−IL-7Rα+ cells in 13.5-dpc fetal livers were separated into αβ+ and αβ− cells, and 5000 cells each were cultured in methylcellulose in 35-mm dishes with recombinant murine M-CSF or GM-CSF. For positive control, Lin−IL-7Rα CD45+ cells were sorted and cultured. Seven days after incubation, the number of colonies were counted.
cell types generated in each thymus organ culture: 1) Ly-5.2 donor cell growth, 2) generation of CD4 mRNA (Fig. 5). In the first row, analyses on a thymus organ culture using Ly-5.2 donor cells were performed. Cells from these lobes were also stained with CD3, CD4, and CD8 to exclude the presence of CD3 CD4 CD8 cells. Approximately 50% of CD3 CD4 cells are CD8, indicating the presence of FILyP cells. In the second row, analyses on a lobe receiving 10 αβ+ cells are presented. This lobe generated both T cells and FILyP cells. The third row represents analyses on a lobe receiving three αβ+ cells. This lobe is positive for FILyP cells, but not T cells. In the lobe presented in the fourth row, we could detect the Ly-5.2+ donor cells with lymphocyte light-scattering pattern. However, because of the absence of CD3 CD4 cells, we judged this lobe negative for FILyP cells. As LTAββ expression is found also in CD4 cells, it is likely that such lobes that are negative in CD3 CD4+ growth, but positive in donor cell growth may contain FILyP cells. The figures are representative of results obtained from 10 independent lobes from two independent experiments for each condition. Lobes received three cells each, and results are similar if three or one hundred cells are placed in a thymic lobe.

>20% of the recovered Lin-IL-7Rα+ cells expressed αβ+ (Fig. 5A). Moreover, ~25% of the Lin-IL-7Rα αβ+ fraction also expressed CD4 (Fig. 5B), whereas no CD4+ cells were present in the initial αβ+ population (data not shown). These in vitro generated Lin-IL-7Rα αβ+ cells expressed LTAββ regardless of CD4 expression (Fig. 5, C and D). This result indicates that Lin-IL-7Rα αβ+ cells are indeed the progenitor of the Lin-IL-7Rα αβ+ FILyP population. RT-PCR analyses revealed that fetal liver Lin-IL-7Rα αβ+ cells expressed both β2 integrin and CD4 mRNA (Fig. 5E).

Does IL-7 affect CD4 or αβ+ gene expression by FILyP? To address this issue, we first sorted Lin-IL-7Rα αβ+ cells from the intestine of 15.5-dpc embryos and cultured them with IL-6 + SCF or IL-7 + SCF for 6 days. The latter population expressed relatively higher level of CD4 than the former (Fig. 6A). However, CD4 expression level was comparable between the FILyP cells with or without 20 µg/ml IL-7 for 24 h after 5 days of culture with IL-6 + SCF (Fig. 6B). Next, FILyP cells from 15.5-dpc intestine were separated according to CD4 expression and cultured with IL-7 + SCF for 7 days without stroma. Expression of IL-7Rα, CD4, and LTAββ was then examined. Only a small number of CD4+ cells developed from the CD4+ population (Fig. 6C). In contrast, approximately one-half of the CD4+ population down-regulated CD4 expression in these culture conditions (Fig. 6D). This down-regulation of CD4 from CD4+ FILyP cells did not affect the expression level of LTAββ (Fig. 6, E and F). The expression level of integrin αβ+ was always comparable among the different in vitro culture conditions (data not shown). These results suggested that IL-7 stimulated both CD4+ and CD4− Lin-IL-7Rα αβ+ cells to express LTAββ on their surface under these culture conditions; however, IL-7 did not stimulate the CD4− FILyP cells to differentiate into CD4+ FILyP cells, nor up-regulate CD4 or integrin αβ+ expression level.

Migration of IL-7Rα+ cells to the antemestenic site of intestines

Our data suggested that Lin-IL-7Rα αβ+ cells were generated in the fetal liver and then migrate to the intestine to be involved in induction of PP anlagen. Consistent with this expectation, the proportion of the Lin-IL-7Rα αβ+ population in the mesenteric region increased before generation of PP anlagen (Fig. 7, upper and middle panels). Histological analyses demonstrated that at 12.5−13.5 dpc, IL-7Rα+ cells first appeared in the mesentery and then subsequently within the intestine from 13.5 dpc (Fig. 7, lower left panel). Interestingly, most IL-7Rα+ cells in the intestine of the 15.5-dpc embryo were distributed preferentially in the antemestenic half of the intestine, where PP anlagen are formed (Fig. 7, lower right panel). Estimated numbers of each organ per embryo are listed in Table III.

Differentiation of the FILyP cells into NK1.1+ cells and CD11c+ cells

We have addressed the derivation and function of Lin-IL-7Rα αβ+ populations that contain FILyP cells. Next, we investigated the fate of Lin-IL-7Rα αβ+ populations in the embryonic intestine. It has been established that both NK and some DC

<table>
<thead>
<tr>
<th>No. of Cells Cultured</th>
<th>Lin-IL-7Rα αβ−</th>
<th>Lin-IL-7Rα αβ+</th>
<th>Lin-IL-7Rα αβ−</th>
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<td></td>
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<td>FILyP cell</td>
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</table>

*Lin-IL-7Rα cells in 12.5 dpc fetal livers were separated into αβ− and αβ+ cells, and 3−100 cells were cultured in a deoxyguanosine-treated thymus lobe from a C57BL/6-Ly5.1 mouse. Fourteen days after incubation, each lobe was assessed for Ly5.2+ donor cell growth, generation of αβ+ and αβ− T cells, and generation of CD1+ CD4+ cells as described in Fig. 4. In the two lobes receiving three αβ− cells, the presence of CD1+ CD4+ CD8+ cells was confirmed.
are derived from lymphoid progenitors (13, 15). In addition, Mebius et al. (17) showed that CD3−CD4−IL-7Rα+αβ7+ cells in mesenteric LN can differentiate into cytotoxic cells and APCs. Thus, we investigated the potential of the intestinal IL-7Rα+αβ7+ population to give rise to NK1.1+ and/or CD11c+ cells.

Lin IL-7Rα+αβ7+ cells were sorted and cultured under various conditions. In the presence of IL-7 and SCF, only a few NK1.1+ cells that coexpress IL-7Ra and αβ7 were induced in the culture (Fig. 8A, upper panels). However, addition of IL-15, a cytokine implicated in the differentiation of NK cells (31, 32), induced a marked increase in NK1.1+ cells (Fig. 8A, lower panels). Interestingly, NK1.1+ cells induced by IL-15 stimulation down-regulated IL-7Ra and αβ7 expression. These results are consistent with previous studies showing that IL-7 induces NK1.1+ progenitors, but their further differentiation requires IL-15 (33). Although IL-15 and SCF did not induce markers for more mature NK cells, such as Ly-49D and DX5 (34, 35), these NK1.1+ cells are competent, as evidenced by cytotoxicity against YAC-1 tumor cells (Fig. 8B).

In the culture of IL-7Ra+ c-Kit+αβ7+ cells stimulated with IL-7 and SCF, no CD11c+ cells could be detected (data not shown). However, a significant number of CD11c− cells were induced when IL-3, TNF, and SCF, which have been demonstrated to be essential for the induction of DC from thymic lymphoid progenitors (36), were added in the culture. As expected, these CD11c+ cells were I-Ahigh, CD86+, CD86+ or high, DEC205low or +, ICAM-1+, Mac1+, and CD86low (Fig. 9A). Of note is that they remain IL-7Rα−, indicating that IL-7Ra expression remains during the initial phase of APC differentiation. As these CD11c+ cells could stimulate allergic CD3−CD4− T cells (Fig. 9B), they are functionally competent in DC activity.

Discussion

FILyP cells, which are responsible for the induction of PP anlagen in neonatal intestine, reside in a population of Lin IL-7Ra+αβ7+ cells in the fetal liver. This population represents an intermediate stage during differentiation of T, FILyP, NK, and DC lineages. We have proposed a model for the induction of PP anlagen (1, 2, 8, 10). The signaling relay between the IL-7Ra induction of LTαβ2+ on the FILyP cells and activation of LTβR on the surrounding intestinal stromal gives rise to distinct PP anlagen that are distinguished from nonstimulated areas by their expression of VCAM-1.
and ICAM-1 (10). We have recently succeeded in isolating and characterizing the VCAM-1- and ICAM-1-expressing mesenchymal cells that are present in the developing intestine of wild-type, but absent from the intestines of PP-defective mutant mice (37). This scenario is in agreement with previous reports showing that mice with null mutations in the genes involved in the IL-7Rα and LTα1β7 signal transduction pathways lack PP (3–6, 38). We have further confirmed that the defect in PP organogenesis in these mutant mice is indeed due to the failure of the formation of VCAM-1/ICAM-1+ PP anlagen, rather than due to the defect in later processes (1, 2, 10).

Induction of LTα1β7 by IL-7Rα signaling

The FILyP cell is part of the Lin−IL-7Rα+CD4+ cell population in neonatal intestine. Lin−IL-7Rα+CD4+− cells, which are freshly isolated from the intestine of 15.5-dpc embryos, express LTα and LTβ mRNA (10). We examined whether IL-7 stimulation can induce LTα1β7 in this population. We found that a combination of IL-6 and SCF promoted the survival of sorted IL-7Rα+ cells without inducing the expression of LTα1β7. Most IL-7Rα+CD4− cells maintained under this condition rapidly expressed surface LTα1β7 as well as LTα and LTβ mRNA upon stimulation with IL-7. This induction is completely blocked by an antagonistic anti-IL-7Rα mAb (data not shown). Taken together, Lin−IL-7Rα+CD4− cells, be it CD4+ or CD4−, are eligible to be termed FILyP cells, although only a portion of this population may be stimulated by the ligand for IL-7Rα in vivo to express LTα1β7. Consistent with this notion, less than 20% of the Lin−IL-7Rα+CD4− cells in the intestine of 16.5-dpc embryo expressed LTα1β7.

Table III. Estimated FLP cell numbers in mesentery and intestine at each day post coitus

<table>
<thead>
<tr>
<th>Gestational Day</th>
<th>Mesentery</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.5</td>
<td>4 ± 2</td>
<td>11 ± 8</td>
</tr>
<tr>
<td>12.5</td>
<td>66 ± 15</td>
<td>96 ± 40</td>
</tr>
<tr>
<td>13.5</td>
<td>3,240 ± 785</td>
<td>3,469 ± 1,822</td>
</tr>
<tr>
<td>15.5</td>
<td>34,037 ± 9,566</td>
<td>65,601 ± 16,980</td>
</tr>
</tbody>
</table>

* Cell numbers of each organ were calculated from the proportion of the Lin−IL-7Rα+CD4− population in the total living cell numbers obtained from the harvested tissue.

Lineage derivation of FILyP cells

Surface expression of IL-7Rα in the absence of lineage markers is in common with lymphoid progenitors (12, 13). Although this observation suggests a close linkage between FILyP cells and lymphocytes, our previous study also demonstrated that the FILyP cell does not express RAG1 nor RAG2 (10), suggesting that this population in the intestine has already diverged from conventional...
lymphocytes. Consistent with this notion, Lin-IL-7Raαβ7+ cells sorted from embryonic intestine could not generate B nor T cells (data not shown). Thus, we addressed the following question: At what stage do FILyP cells diverge from the differentiation pathway of T and B lymphocytes?

Lin- c-Kit-IL-7Raαβ4+ progenitors can give rise to B, T, NK, and DC (12–15, 39). Although this population may contain colony-forming progenitors of myelomonocytic lineage at a low frequency, most are committed progenitors of lymphoid lineages. In this study, we further dissected this population into αββ7+ and αββ7+ populations and found that B-potential is lost in the αββ7+ population, whereas T-potential remains to some extent. Hence, T cell progenitor and FILyP cell diverge later than the progenitors with B-potential. This is consistent with our findings that there are T-committed or B-committed lymphoid progenitor cells in fetal liver (40). As demonstrated in Table II, αββ7+ cells showed a greater tendency toward giving rise to FILyP cells whereas αββ7+ cells generated both FILyP and T cells equally. Moreover, our results demonstrated that fetal liver Lin-IL-7Raαβ7+ cells quickly differentiated into Lin-IL-7Raαβ7+ cells, indicating a different order of differentiation from Lin-IL-7Raαββ7+ to Lin-IL-7Raαβ7+ populations. In addition, by means of RT-PCR analysis, we found that fetal liver Lin-IL-7Raαβ7+ cells are already expressing β7 integrin or CD4 mRNA. This result indicated at least a part of the Lin-IL-7Raαβ7+ cells in fetal liver is committed to the Lin-IL-7Raαβ7+CD4+ cells. As Lin-IL-7Raαβ7+ cells could differentiate to NK and DC in vitro, our result is consistent with those of Zhang et al., who showed that NK or DC cells were induced from fetal liver cells using a stromal cell-dependent culture system (41). In addition, we have confirmed cells expressing NK or DC markers differentiating from fetal liver Lin-IL-7Raαβ7+ or Lin-IL-7Raαββ7+ cells (data not shown). Based upon these lines of evidence, we propose a model summarized in Fig. 9. In this model, the commitment to the FILyP cell pathway occurs in the fetal liver, and is thought to progress in the following order: loss of B-potential, expression of αβ7 integrin, loss of T-potential, and migration to the intestine.

**Relationship between T/FILyP cells and T/NK progenitors**

The T/NK progenitor in fetal thymus (14), which was shown to be c-Kit+/IL-7Ra+CD44+Thy-1−NK1.1−, appears similar to the liver-derived population that gives rise to T and FILyP cells. Because αββ7+ cells from fetal liver or intestine contained both Thy-1-positive and Thy-1-negative cells and were NK1.1− (10) (data not shown), liver-derived αββ7+ cells may represent a more immature population. Nonetheless, when IL-7Raαβ7+ cells were stimulated with IL-15 in the thymus organ culture system, all lobes containing FILyP cells also contained NK1.1+ cells (Kawamoto, unpublished observation). Moreover, Lin-IL-7Raαβ7+ cells in the intestine can give rise to NK cells in the culture. Thus, it is likely that T/FILyP and T/NK progenitors represent the same population. Kumar and colleagues (13) identified NK progenitors in the c-Kit+IL-7Ra+NK1.1− population of the bone marrow. Due to the difficulty in distinguishing multipotent progenitors from committed progenitors, the presence of T/NK progenitors in hematopoietic tissues has not been documented, although their presence in the thymus is well established (13, 14). In this respect, our results raise the possibility that extrathymic hematopoietic tissues are also the sites in which T/NK progenitors are generated. Obviously, this notion does not exclude thymic development of T/NK progenitors.

**Fate of FILyP cells in ontogeny**

The Lin-IL-7Raαβ7+ population containing FILyP cell then migrates to the intestine through the mesentery. Our observation that the proportion of αββ7+ cells increases in the intestine suggests continuous recruitment of FILyP cells from fetal liver, although it is also possible that αββ7+ cells may preferentially proliferate in the intestine. We also showed that they migrate preferentially to the antimesenteric site of the developing intestine, which is the site of PP induction. This observation implies the presence of an active process regulating the directed migration of
the Lin<sup>-</sup>IL-7Ra<sup>+</sup>α<sub>d</sub>β<sub>7</sub><sup>+</sup> population. Mebius et al. (42) have previously shown that the adhesion between mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and α<sub>d</sub>β<sub>7</sub> integrin plays an important role in the migration and/or proliferation of CD4<sup>+</sup>CD3<sup>+</sup>α<sub>d</sub>β<sub>7</sub><sup>+</sup>IL-7Ra<sup>+</sup> cells to the developing LN, using injection of anti-MAdCAM-1 mAb. Because the cellular origin seems to be basically the same between our FILyP cells and their CD4<sup>+</sup>CD3<sup>+</sup>α<sub>d</sub>β<sub>7</sub><sup>+</sup>IL-7Ra<sup>+</sup> cells, α<sub>d</sub>β<sub>7</sub> integrin may play some roles in FILyP cell migration to the PP anlagen. However, we found no difference in the PP anlagen formation with the blockade of MAdCAM-1 and α<sub>d</sub>β<sub>7</sub> integrin adhesion by injection of anti-MAdCAM-1 mAB MECA367 (43). Recently, we found that PP-specific mesenchymal cells expressing VCAM-1/ICAM-1 produce the chemokines B lymphocyte chemoattractant and EBI-ligand chemokine during development (37). It was reported that B lymphocyte chemoattractant-defective or CXCR5-defective mutant cells have a partial defect in PP organogenesis (44, 45), and that CXCR5 is expressed in FILyP cell population (10, 17). Combination of MAdCAM-1/integrin α<sub>d</sub>β<sub>7</sub> interaction and the function of those chemokines may play a pivotal role in FILyP cell migration to the periphery. The molecular nature of FILyP cell migration to the intestine should be studied in the future. Because the intestinal Lin<sup>-</sup>IL-7Ra<sup>+</sup>α<sub>d</sub>β<sub>7</sub><sup>+</sup> population lacks the ability to differentiate to T cells, this population undergoes irreversible differentiation before migration to the intestine. After being seeded in the antimesenteric site of intestine, a portion of Lin<sup>-</sup>IL-7Ra<sup>+</sup>α<sub>d</sub>β<sub>7</sub><sup>+</sup> cells comes into contact with cells expressing the ligand for IL-7Ra<sup>+</sup>, thereby inducing LTα<sub>7</sub>β<sub>2</sub> expression, resulting in the induction of the PP anlagen (10).

Induction of PP anlagen may not be the sole function of FILyP cells, as they can further differentiate into cytotoxic NK1.1<sup>+</sup> and Ag-presenting CD11c<sup>+</sup> populations under appropriate conditions. Consistent with the previous report that NK progenitors could be induced in bone marrow cultures with IL-7 and SCF (33), this cytokine combination induced a small proportion of NK1.1<sup>+</sup> cells in the IL-7Ra<sup>+</sup> population. This NK1.1<sup>+</sup> population maintained expression of IL-7Rα, which was then down-regulated upon addition of IL-15. Thus, IL-7 can support differentiation of FILyP cells to c-Kit<sup>+</sup>IL-7Ra<sup>+</sup>NK1.1<sup>+</sup> stage, but IL-15 is required for further differentiation. Although IL-15-induced cytotoxic NK1.1<sup>+</sup> cells did not express mature NK markers such as Ly-49D nor DX5 (34, 35), they were functionally competent (Fig. 9). This is consistent with recent reports demonstrating a difference between adult and fetal NK cells in the ability to express Ly-49 family molecules (14, 46, 47).

From the present results, FILyP cells are shown to give rise to functional APCs expressing CD11c, I-A, CD80, CD86, ICAM-1, MAC-1, and CD8α upon IL-3 and TNF-α stimulation. However, over a 4-day period in culture, the expression of IL-7Ra<sup>+</sup> was not down-regulated. DC has been classified into Mac-1<sup>-</sup>CD8α<sup>-</sup>DEC205<sup>+</sup> lymphoid DC and Mac-1<sup>+</sup>CD8α<sup>-</sup>DEC205<sup>-</sup> myeloid DC in situ (15, 48). The surface phenotype of DC described in this study does not fit either phenotype, suggesting that they may represent a novel population or an immature stage of differentiation. Recent identification of CD8α<sup>-</sup> and Mac-1 double-positive DC in the spleen (49) supports the former possibility that the FILyP cell-derived DC constitute a subset of DC in peripheral lymphoid organs.

**FIGURE 10.** A model for the differentiation pathway of the FILyP cell. In this model, FILyP cells are derived from the IL-7Ra<sup>+</sup> c-Kit<sup>+</sup>α<sub>d</sub>β<sub>7</sub><sup>+</sup> population, which also gives rise to T and B cells, but not myeloid cells. As it was suggested that common lymphoid progenitors are not found in the fetal liver (17, 34), this population may be a mixture of T- and B-committed progenitors. The FILyP lineage diverges from the B cell lineage before expression of α<sub>d</sub>β<sub>7</sub> integrin, while T-potential remains in the α<sub>d</sub>β<sub>7</sub><sup>+</sup> population. In the liver-derived Lin<sup>-</sup>IL-7Ra<sup>+</sup> c-Kit<sup>+</sup>α<sub>d</sub>β<sub>7</sub><sup>+</sup> population, more cells restricted to FILyP cells are present than those with T-potential. T-potential is lost completely from this population during their migration from fetal liver to the mesenteric tissues. After migration to the intestine, a portion of FILyP cells is activated by the ligand for IL-7Ra<sup>+</sup> and involved in induction of PP anlagen by expressing LTα<sub>7</sub>β<sub>2</sub>. During this process, expression of IL-7Ra<sup>+</sup> and α<sub>d</sub>β<sub>7</sub> integrin is maintained. In this model, CD4<sup>+</sup> expression is thought to associate with the divergence from NK lineage. If the appropriate signals are available in the microenvironment, this population can further differentiate to NK or DC lineages.
The differentiation process, which occurs after acquisition of the FILyP cell phenotype, is depicted in Fig. 10. In this model, FILyP cells are the progenitors of NK and DC lineages, the potential to the former decreasing during differentiation from CD4+ to CD4− cells. This scheme includes our unpublished observation that the potential to give rise to NK1.1+ cells decreased during differentiation of Lin−IL-7Rα−βc−β7−CD4+ to Lin−IL-7Rα−α4−β7−CD4+. Our model conflicts with those of other groups (12, 14, 39). This could be due to the presence of multiple pathways of NK and DC development. In fact, our previous study on Id2+/− mice indicates Id2-dependent and Id2-independent pathways of NK cell differentiation (11). DC can differentiate even from a pro-B cell population (29). It is also likely that these discrepancies reflect differences in stromal cell activity among organs. NK cell differentiation has been thought to occur in the central lymphoid organs such as bone marrow and thymus (13, 14). Recently, Iizuka et al. (50) reported that NK lineage differentiation is impaired in Lto−/− mice with defects in both the microenvironment and hematopoietic lineage. This suggests that establishment of the architecture of lymphoid tissue and generation of NK cells in the same tissue are concurrent processes in which Ltoαβ−positive cells play major roles. If so, this notion could be extended to peripheral lymphoid tissues, in which Ltoαβ−positive cells are required for organogenesis. There is a strong correlation between defects in PP/LN organogenesis and absence of functional NK cells (11, 51, 52), which is consistent with an idea of a common progenitor for FILyP and NK cells. However, there also appears to be a dichotomy in LN and PP formation, as PP can form in the absence of LN as in Osteopetrosis mice (37). However, there also appears to be a dichotomy in LN and PP formation, as PP can form in the absence of LN as in Osteopetrosis mice (37). Therefore, the notion could be extended to peripheral lymphoid tissues, in which Ltoαβ−positive cells serve as a useful marker for dissecting both common and organ-specific processes.

Acknowledgments

We are grateful to Mizuho Satoh for technical assistance, to Dr. Ikawa and Dr. Ohmura for their advice on cytotoxic and cell proliferation assays, and to Dr. S. Frazer for critical reading of the manuscript.

References


