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*J Immunol* 1998; 160:4801-4809; 
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Ftlt3 Ligand Plus IL-7 Supports the Expansion of Murine Thymic B Cell Progenitors That Can Mature Intrathymically

Hilary J. McKenna¹ and Philip J. Morrissey

Flt3 ligand (ftlt3L) has potent effects on hemopoietic progenitors, dendritic cells, and B lymphopoiesis. We have investigated the effects of ftlt3L on intrathymic precursors. The addition of ftlt3L + IL-7 to lobe submersion cultures of murine fetal thymic lobes resulted in the expansion of an immature population of Thy-1low, CD44high, HSAhigh cells. This population contained cells with precursor activity, as determined by their capacity to repopulate deoxyguanosine-treated fetal thymic lobes. Upon reentry to the thymic lobe, ftlt3L + IL-7-cultured Thy-1low, CD44high, HSAhigh cells underwent expansion and differentiation into B cells. Two weeks after fetal thymic organ culture following thymic lobe reconstitution, intrathymic cells were Thy-1−, B220+ and subset was slgM+. The intrathymic B cells shared features of adult thymic B cells, including CD5 expression and proliferative responses to IL-4 + IL-5 + CD40 ligand, but not to LPS or soluble anti-IgM. Ig production was noted upon stimulation with IL-4 + IL-5 + LPS and IL-4 + IL-5 + CD40 ligand. In conclusion, we have demonstrated that ftlt3L + IL-7 supports the expansion of a subset of progenitors present in the fetal thymus. The cultured progenitors can repopulate a fetal thymic lobe and develop into mature functional B cells, demonstrating that the fetal thymus is able to support B cell as well as T cell development. The Journal of Immunology, 1998, 160: 4801–4809.

Hemopoietic progenitors from the embryonic liver, and later, adult bone marrow enter the thymus, and after a period of intrathymic development, mature T cells with rearranged γδ and αβ genes are generated (reviewed in Refs. 1–4). The nature of the progenitor that seeds the thymus is unclear, but reports suggest that in addition to progenitors for the T cell lineage, there are progenitors for the B lymphoid (5), NK (6), and macrophage lineages (5, 7) in the murine fetal thymus, and for the B lymphoid (8–10) and dendritic cell (11, 12) lineages in the murine adult thymus, indicating that the progenitors that enter the thymus are not all committed to the T cell lineage.

Mature B cells are present in the thymus of mice, where they comprise approximately 1% of the total cells (reviewed in Ref. 13), and are postulated to have a role in the deletion of autoreactive T cells (14, 15). During murine ontogeny, mature B cells expressing surface IgM (slgM)² are detected in the fetal thymus on approximately day 14 (16). Thymic B cells differ from conventional splenic B cells in that they express relatively low levels of B220 and MHC class II on their surface (17), although they express cell slgM at levels comparable with splenic B cells (17). Unlike splenic B cells, thymic B cells express CD11b (Mac-1) and a large proportion are CD5− (17), which are features of peritoneal cavity Ly-1 B cells. However, adoptive transfer studies have shown that thymic B cells can be derived from both bone marrow- and fetal liver-derived progenitors (18), unlike the peritoneal Ly-1 B cells that appear to be a self-renewing subset of B cells derived solely from fetal liver progenitors (19, 20). Purified thymic B cells do not proliferate in response to LPS or anti-IgM + IL-4 (21), although these factors deliver strong proliferative signals to splenic B cells, but thymic B cells proliferate in response to CD40 ligand (CD40L) + IL-10 (22). The combined stimulus of CD40L + IL-4 also induces production of soluble IgM and IgG at levels comparable with those produced by splenic B cells (22). The ability of thymic stromal cell lines to support B cell maturation in vitro has been described (10, 23), and it was reported recently that the B cells found in the adult thymus can develop within that environment from a B cell progenitor, described as B220medium, slgM−, CD43− (9), suggesting that thymic B cells develop in situ. However, the process of intrathymic B cell development is not understood, and the role of cytokines that may influence intrathymic B cell development has not been defined.

Flt3 ligand (ftlt3L) is a hemopoietic growth factor that has effects on progenitors of both lymphoid and myeloid lineages (reviewed in Ref. 24). We have examined the role of ftlt3L in T cell development using murine fetal thymic organ culture (FTOC) as a model. We report in this work that the combination of ftlt3L + IL-7, but neither factor alone, supports the expansion of primitive thymocytes in lobe submersion cultures (LSC). These primitive cells retain their capacity to reenter an alymphoid (deoxyguanosine (dGuo)-treated) fetal thymic lobe, where they undergo expansion and development. However, the progeny of these ftlt3L + IL-7-expanded progenitors were not T cells; instead, we observed B cell development in the thymus. The B cells shared characteristics described for intrathymic B cells, including CD5 expression, proliferative responses to CD40L (but not LPS or anti-IgM stimulation), and soluble Ig production upon stimulation with cytokines. We conclude that the fetal thymic environment can support B cell lymphopoiesis, starting with an intrathymic precursor that is responsive to ftlt3L + IL-7, and ending with a mature, functional B220+, IgM+B cell.

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² Abbreviations used in this paper: slgM, surface IgM; CD40L, CD40 ligand; CD40LT, CD40 ligand trimer; sIgM, deoxyguanosine; ftlt3L, ftlt3 ligand; FTOC, fetal thymic organ culture; HSA, heat-stable Ag; SLF, lobe submersion culture; rHu, recombinant human; SLF, steel factor; rHu, recombinant human.

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Received for publication October 21, 1997. Accepted for publication January 21, 1998.

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0022-1767/98/$02.00


**Materials and Methods**

**Animals**

Timed pregnant C57BL/6 mice were purchased from either The Jackson Laboratory (Bar Harbor, ME) or Harlan Sprague-Dawley (Indianapolis, IN). Timed pregnant C57BL/6 Ly-5.2 mice were purchased from Frederick Cancer Research Institute (Frederick, MD). In addition, timed pregnant C57BL/6 and C57BL/6 Ly-5.2 mice were produced at ImmuneX (Seattle, WA). Adult C57BL/6 females (6–8 wk old) were purchased from The Jackson Laboratory.

**Lobe submersion cultures**

Gestational day 15 fetal thymic lobes were cultured in 24-well plates (Costar, Cambridge, MA) in 1 ml DMEM (Life Technologies, Grand Island, NY) containing 10% FBS (Intergen, Purchase, NY), 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine (JRH Biosciences, Lenexa, KS), y-2 × 10⁻⁵ M 2-ME, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies) (complete DMEM). Day 15 lobes were cultured at a density of three lobes per well in a 37°C, humidified, 10% CO₂ incubator. Cytokines were produced and purified at ImmuneX and used at the following concentrations: rhuIL-7 (100 ng/ml) and Chinese hamster ovary cell line-derived rhuFL3L (100 ng/ml).

**Fetal thymic organ culture**

Fetal thymic lobes were cultured at the liquid/air interface in six-well plates (Costar) containing 0.4-μm membrane inserts, at six to eight lobes per well. Media, as described for the LSC, were placed in the bottom of the well (1.5 ml). No exogenous cytokines were added. The plates were incubated in a 37°C, humidified, 10% CO₂ incubator. Lobes were removed from the membranes with fine forceps, and cell suspensions were prepared by gently pressing the thymic lobes beneath a hemocytometer coverslip to release the thymocytes. Viable cell counts were determined by trypan blue exclusion.

**Proliferation assays**

Cell suspensions of freshly isolated fetal thymocytes were obtained by gently pressing fetal thymic lobes beneath a coverslip to release the thymocytes. The cells were then passed over nylon filters to remove large clumps of cells. Proliferation assays were performed in Linbro 96-well round-bottom plates (ICN Biomedicals, Aurora, OH). A quantity amounting to 3 × 10⁶ cells/well was plated in triplicate in a final volume of 100 μl of media, as described for LSC. Cells were incubated for 3 days and then pulsed for 8 h with 2 μCi/well of tritiated thymidine (Amersham, Arlington Heights, IL). Samples were harvested and counted using Geiger aboradicles (ICN Biomedicals, Aurora, OH). Day 15 fetal thymic lobes were cultured in 24-well plates (Costar) containing 0.2 μg/ml of complete DMEM (106 cells/well). Wells were supplemented with combinations of rmuIL-4 (10 ng/ml), rmuIL-5 (10 ng/ml), rmuCD40LT (3 μg/ml), and LPS (25 μg/ml). After 7 days, the supernatants were harvested and the presence of IgG1 and IgE was detected by ELISA, as previously described (27). IgM was detected using the same sandwich ELISA protocol (27). The plates were coated with goat anti-mouse IgM (Southern Biotechnology Associates), and for detection of IgM, goat anti-mouse IgG horseradish peroxidase (Southern Biotechnological Associates) was used. IgM concentrations were estimated using purified mouse IgM as a standard (Southern Biotechnological Associates).

**Results**

The effect of flt3L in LSC of fetal thymus

mRNA for flt3 receptor (flt3R) has been detected in the earliest thymocytes (28), and it has previously been reported that flt3L, both as a single agent and in combination with IL-7, stimulates the proliferation of fetal thymocytes in vitro (29). In addition, when flt3L was added to a human thymic stromal culture supplemented with IL-12, the generation of T cells from CD34+ cells was augmented (30). These reports suggest that flt3L may have a role in early events in T cell development. We have noted previously the effect of the addition of IL-7 and SLF to LSC of intact fetal thymus (31). The addition of either IL-7 or SLF to LSC promoted thymocyte growth and SLF synergized with IL-7, resulting in the expansion of immature thymocytes (CD3⁺, CD4⁺, CD8⁺) (31). We therefore examined the effect of the addition of flt3L to LSC of day 15 fetal thymi. When flt3L alone was added to LSC, no expansion of fetal thymocytes was noted after 7 days of culture (Fig. 1A). Only 10% of the cells from day 15 lobes survived 7 days in LSC with flt3L, and the cells were comprised mainly of adherent stromal cells, similar to the yield from cultures that develop in the absence of added growth factors (Fig. 1A). Addition of IL-7 alone to LSC of day 15 fetal thymi resulted in an increase in cell yield compared with freshly isolated thymus yields (average 1.4-fold), or an average 15-fold compared with LSC with no cytokines. The addition of flt3L + IL-7 resulted in an average 1.9-fold increase in cell yield compared with freshly isolated thymus yields, or an average 20-fold increase in cell yield compared with LSC with no cytokines (Fig. 1A). Phenotypic analysis of cells harvested from the flt3L + IL-7 cultures revealed an increase in the proportion of cells with the phenotype Thy-1low, HA⁺high (Fig. 1B). After 7 days in LSC with IL-7 alone, 4.3% of the cells were Thy-1low, HA⁺high compared with 10% when flt3L was added with IL-7. Although LSC supplemented with flt3L alone resulted in the highest proportion of cells with this phenotype (20.1%), when the absolute number of the Thy-1low, HA⁺high cells in the cultures was calculated, only cultures supplemented with flt3L + IL-7 resulted in an increase in Thy-1low, HA⁺high cell numbers (average 1.8-fold compared with no culture) (Fig. 1C). Analysis of the Thy-1low.
HSA\textsuperscript{high} cells from freshly isolated thymi revealed the following phenotype: CD44\textsuperscript{high}, CD11b\textsuperscript{2}, B220\textsuperscript{2}, and a subset was IL-2R\textalpha\textsuperscript{1} (Fig. 1D), CD3\textsuperscript{2}, CD4\textsuperscript{2}, CD8\textsuperscript{2}, \alpha\beta TCR\textsuperscript{2}, \gamma\delta TCR\textsuperscript{2}, IL-2R\beta\textsuperscript{2}, NK1.1\textsuperscript{2}, sIgM\textsuperscript{2}, sIgD\textsuperscript{2}, and Gr-1\textsuperscript{2} (data not shown). This phenotype is consistent with that of early pro-T and pre-T cells (2, 3). After 7 days in LSC supplemented with either IL-7 or
flt3L + IL-7, the Thy-1low, HSAhigh cells were analyzed for the expression of the same cell surface markers. The only changes noted in both groups compared with the freshly isolated Thy-1low, HSAhigh cells were the loss of IL-2R expression and the up-regulation of the integrin CD11b (Fig. 1D). CD44 levels remained high, and B220 was not detected when compared with cells incubated with isotype-matched control Abs. The data suggested that the combination of flt3L + IL-7, when added to intact fetal thymic LSC, induced the expansion of a population of thymocytes with an immature phenotype.

**flt3L plus IL-7 induces proliferation of immature thymocytes**

We next examined the effect of flt3L and IL-7 on freshly isolated fetal thymocytes in short-term proliferation assays. Day 15 thymocytes were sorted on the basis of Thy-1 and HSA expression into two groups: Thy-1low, HSAhigh, and Thy-1high cells (which express variable levels of HSA (Fig. 1B)). These thymocyte subsets were tested for their proliferative response to flt3L and IL-7 alone and flt3L + IL-7 at concentrations determined to be effective (data not shown). Flt3L alone failed to induce proliferation of fetal thymocytes (Fig. 2). IL-7 alone induced proliferation of Thy-1low, HSAhigh, and Thy-1high cells, as well as the unfractionated thymocytes (presort) (Fig. 2). Unfractionated thymocytes responded synergistically to the combination of flt3L + IL-7, and this synergistic response was observed in the Thy-1low, HSAhigh fraction of cells, but not the Thy-1high fraction. The population of Thy-1low, HSAhigh cells expanded in LSC with flt3L + IL-7 (Fig. 1C) also responded to this cytokine combination in a short-term proliferation assay.

**B cell precursors are contained within the population expanded by flt3L + IL-7 in LSC**

With the exception of the expression of the integrin CD11b, the phenotype of the thymocytes that were expanded in LSC with flt3L + IL-7 (Thy-1low, HSAhigh cells) suggested that these cells may be relatively primitive progenitor cells (2, 3). To determine whether these cells had progenitor potential, Thy-1low, HSAhigh cells were isolated by cell sorting, and tested for their capacity to repopulate dGuo-treated day 15 fetal thymic lobes. DGuo has been shown to deplete fetal thymus of all lymphoid cells, but leaves intact the stromal components of the thymic lobe (25, 26). First, 1) unfractonated, 2) Thy-1low, HSAhigh, and 3) Thy-1high cells from freshly isolated day 15 fetal thymus were cocultured in hanging drops with dGuo-treated lobes for 48 h. The lobes were returned to culture on transmembrane well inserts at the air-liquid interface (FTOC) for 10 to 15 days in the absence of exogenous cytokines. Cells from each group repopulated dGuo-treated lobes (Table I). Thy-1low, HSAhigh thymocytes had the greatest expansion potential within the thymus on a per cell basis. Next, the same cell populations were isolated from fetal thymus cultured in LSC supplemented with IL-7 alone or flt3L + IL-7, and their repopulation potential was determined in the same manner. Cells from the IL-7 cultures consistently showed poor repopulation ability (Table II), suggesting that even though Thy-1low, HSAhigh cells could be isolated from the cultures supplemented with IL-7, these cells did not repopulate dGuo-treated lobes.

**Table I. Noncultured fetal thymocytes repopulate dGuo-treated fetal thymic lobes**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Thymocyte Population</th>
<th>Cells Cocultured with dGuo-Treated Lobe (×10^-4)</th>
<th>Yield/Lobe (×10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td>No cells</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Unfractionated</td>
<td>20.0</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Thy-1low, HSAhigh</td>
<td>1.6</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>Thy-1high</td>
<td>30.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>No cells</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Thy-1low, HSAhigh</td>
<td>1.0</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Thy-1high</td>
<td>1.0</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Thy-1high</td>
<td>10.0</td>
<td>5.1</td>
</tr>
</tbody>
</table>

a Thy-1low, HSAhigh, and Thy-1high fetal thymocytes were isolated by cell sorting from day 15 embryonic thymus. Thymocytes were cocultured in hanging drop cultures for 48 h with day 15 fetal thymic lobes that had been treated for 5 days with dGuo (see Materials and Methods) (8–12 lobes per group). Lobes were returned to FTOC after 13 days (experiment 1) or 10 days (experiment 2) in FTOC, lobes were harvested, cell suspensions were prepared, and the yield of viable cells per lobe was determined.
isolated unfractionated or Thy-1 high fetal thymocytes also con-
lobes repopulated with freshly isolated Thy-1low, HSA high cells
that were expanding intrathymically. After 13 days of FTOC,
with the dGuo-treated lobes.

Less than 20% were Ia^+ cells that could be further defined by CD4 and

A dose response was performed to determine how many Thy-1low, HSA^high cells from the flt3L + IL-7 cultures were able to repopulate the dGuo lobes and undergo expansion (Table II).

A dose response was performed to determine how many Thy-1low, HSA^high cells from LSC supplemented with flt3L + IL-7 were required to ensure lobe repopulation. Cell numbers ranging from 1.6 × 10^4 to 2 × 10^5 were cocultured with dGuo-treated lobes, then returned to FTOC (Fig. 3). After 14 days, cell yields were determined. As few as 3.2 × 10^3 Thy-1low, HSA^high cells resulted in a low level of repopulation, and repopulation appeared to plateau when 1 × 10^4 Thy-1low, HSA^high cells were cocultured with the dGuo-treated lobes.

We next examined the repopulated lobes to characterize the cells that were expanding intrathymically. After 13 days of FTOC, lobes repopulated with freshly isolated Thy-1low, HSA^high cells contained Thy-1high cells that could be further defined by CD4 and CD8 expression (Fig. 4A). Similarly, lobes repopulated by freshly isolated unfractionated or Thy-1high fetal thymocytes also contained Thy-1high cells expressing CD4 and CD8 (data not shown). However, when the lobes repopulated by the Thy-1low, HSA^high cells isolated from the flt3L + IL-7 LSC were examined after 13 days of FTOC, no Thy-1high, CD4^+, CD8^+ cells were detected. Instead, the intrathymic cells were B220^+. suggesting that they may be developing B cells (Fig. 4B). In addition, the B220^+ cells had retained HSA expression, but lost CD11b expression. Significant numbers of NK1.1^+ or Gr-1^+ cells were not detected in any of the repopulated lobes (Fig. 4, A and B).

We conclude that a population of Thy-1low, HSA^high cells present in the day 15 fetal thymus expanded in response to flt3L + IL-7, and within this population of cells were thymic B cell precursors (B220^+), which retained their capacity to reenter a thymic lobe and develop into B220^+ cells intrathymically.

Intrathymic B cell development

Further characterization of the intrathymic B220^+ cells was performed using immunofluorescent staining and flow cytometry (Fig. 5). Ten to fifteen days after lobe repopulation, approximately 30% of the B220^+ cells were slgM^+ and 15 to 30% were CD5^+. Less than 20% were Ia^+, and those that did express Ia expressed relatively low levels. slgD and CD23 were generally not detected on the B220^+ cells.

To confirm that the developing B220^+ cells were indeed derived from the Thy-1low, HSA^high progenitors isolated from the flt3L + IL-7-supplemented LSC, and not from an intrathymic cell that survived the dGuo treatment, repopulation experiments were performed using thymic lobes from C57BL/6 Ly-5 congenic mice. Fetal thymic lobes from C57BL/6 Ly-5.1 embryos were cultured in flt3L + IL-7 for 7 days, and the Thy-1low, HSA^high cells were

FIGURE 3. Dose response of fetal thymic lobe repopulation by Thy-1low, HSA^high thymocytes isolated after LSC in flt3L + IL-7. Thy-1low, HSA^high thymocytes from 7-day LSC supplemented with flt3L + IL-7 were isolated by cell sorting. Numbers ranging from 1.6 × 10^2 to 2 × 10^4 were cocultured with dGuo-treated day 15 fetal thymic lobes for 48 h, then the lobes were returned to fetal thymic organ culture (FTOC). After 14 days, lobes were harvested and cellularity was determined. Each group included 10 to 15 lobes. The grey bar represents the yield per lobe cocultured with no cells.

FIGURE 4. A. Repopulation of dGuo-treated lobes with Thy-1low, HSA^high cells isolated from freshly isolated and flt3L + IL-7-cultured fetal thymic lobes. A, Thymocytes were isolated from day 15 fetal thymic lobes, and Thy-1low, HSA^high cells were isolated by cell sorting. A quantity amounting to 1.6 × 10^5 cells was cocultured with dGuo-treated lobes for 48 h, then the lobes were returned to FTOC. After 13 days, cells were isolated from the lobes and the phenotype was examined by flow cytometry. The yield per lobe was 8.2 × 10^4 cells. The percentage of cells in each quadrant is shown. Quadrant markers were set based on the profiles obtained with isotype-matched control Abs. B, Thymocytes were isolated from day 15 fetal thymic lobes that were cultured for 7 days with flt3L + IL-7 in LSC, Thy-1low, HSA^high cells were purified by cell sorting, and 1.6 × 10^4 were cocultured with dGuo-treated lobes for 48 h, then returned to FTOC (as described in A). After 13 days, cells were isolated from the lobes and the phenotype was examined by flow cytometry. The yield per lobe was 4.8 × 10^4 cells.
isolated by cell sorting. The Thy-1 low, HSA high cells were cocultured in hanging drops with dGuo-treated thymic lobes isolated from C57BL/6 Ly-5.2 embryos. The lobes were returned to FTOC, and after 10 to 14 days, the lobes were analyzed for Ly-5.1 and Ly-5.2 expression (Fig. 6). In each of three separate experiments, the intrathymic cells were Thy-1^2, B220^1, and Ly-5.1^1. This demonstrated that the progenitors that were giving rise to the development of B cells intrathymically were derived from the Thy-1 low, HSA high cells isolated from flt3L and IL-7 LSC, and not from the dGuo-treated lobes.

**Intrathymic B cell proliferative responses and Ig production**

The ability of the intrathymic B cells to proliferate and produce Ig in response to various B cell stimuli was examined to determine their functional potential. Thy-1 low, HSA high cells from flt3L + IL-7 LSC were isolated and cocultured with dGuo-treated lobes, then returned to FTOC. After 10 to 14 days, the lobes were harvested, and the intrathymic cells were isolated. A fraction of the cells was removed and checked for phenotype by immunofluorescent staining and flow cytometry. Incubation with B220 and Thy-1 Abs revealed that all of the cells were B cells (data not shown). The proliferative responses to the B cell stimuli LPS, soluble anti-IgM, and the combination of IL-4 and IL-5 and CD40LT were examined. Adult-derived splenocytes were used as a source of mature B cells in the same assays for comparison. As expected, splenocytes proliferated in response to each of the stimuli, with IL-4 and IL-5 and CD40LT inducing the greatest level of proliferation (Fig. 7). In contrast, the fetal thymic-derived B cells did not proliferate in response to LPS or anti-IgM, but did respond to IL-4 and IL-5 and CD40LT (Fig. 7).

In addition to proliferative responses, soluble Ig production was also examined. Repopulated lobes were harvested, and a cell suspension of the B cells was obtained. The B cells were cultured for a further 7 days in the presence of various B cell stimuli. The supernatants were harvested and tested by ELISA for the presence of soluble IgM, IgG1, and IgE. Adult-derived splenocytes were also cultured for 7 days in the presence of the same factors for comparison. No Ig was produced in the media-alone cultures (Table III). IL-4 + IL-5 plus either LPS or CD40LT stimulated the production of all three Ig isotypes from both the splenic B cells and
and when CD4 low cells are transferred i.v. into irradiated animals, triplicates and representative of three separate experiments.

Overall, the splenic B cells produced more Ig than the thymic B cells (9). In addition, the observations that progenitors from the nonirradiated neonatal thymi, was able to generate mature thymic B cells (9). In addition, the observations that progenitors from the nonirradiated neonatal thymi, was able to generate mature thymic B cells (9). In addition, the observations that progenitors from the nonirradiated neonatal thymi, was able to generate mature thymic B cells (9).

Discussion

The presence of progenitors in the fetal and adult thymus with the potential to develop into lineages other than the T lymphoid lineage has been described (5–12); it has been shown that the adult thymus, in addition to supporting T cell development, can also support dendritic cell development (12). The most primitive progenitors isolated from the thymus is capable of supporting B cell lymphopoiesis through a functional sIgM + B cell in vitro. These progenitors were contained within the Thy-1low, HSAhigh population of thymocytes, a fraction that by phenotype appears relatively undifferentiated (CD44high, CD3−, CD4−, CD8−, TCR−) (Fig. 1, B and D). When intact day 15 fetal thymic lobes were cultured in the presence of flt3L plus IL-7, but not with either factor alone, the population of Thy-1low, HSAhigh cells was expanded (Fig. 1, B and C). The expanded Thy-1low, HSAhigh cells remained CD44high, CD3−, CD4−, CD8−, TCR−, lost IL-2Rα expression, and gained high levels of CD11b expression. Freshly isolated Thy-1low, HSAhigh cells repopulated dGuo-treated lobes and generated T cells. However, after culture for 1 wk in LSC supplemented with flt3L plus IL-7, Thy-1low, HSAhigh cells repopulated dGuo-treated lobes and generated B cells. This implies that the signals delivered by flt3L plus IL-7, in the context of an intact fetal thymic lobe, either support the survival of B cell progenitors, but not T cell progenitors present in the lobe, or flt3L plus IL-7 directs commitment of a bipotent or multipotent progenitor cell present in the thymus to the B cell lineage. The ensuing intrathymic B cell development did not require concomitant T cell development, as we failed to see T cell repopulation from the cultured progenitors (Figs. 4B and 5). Thymic stromal cells are a rich source of cytokines (reviewed in Ref. 32), including flt3L and IL-7 (29, 32, 33), and it is difficult to know whether endogenously produced cytokines in addition to flt3L plus IL-7 played a role in supporting survival of the B cell progenitors. It is also unclear what role the absence of extrathympic influences including hormones may have on the development of cells in the thymus in vitro. Extrathympic influences have been shown to influence T cell development via their interaction and effect on thymic epithelial cells (reviewed in Ref. 34).

**Table III. Immunoglobulin produced by B cells isolated from repopulated fetal thymic lobes**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cells</th>
<th>IgM (μg/ml)</th>
<th>IgG1 (μg/ml)</th>
<th>IgE (μg/ml)</th>
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</thead>
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<tr>
<td>Medium</td>
<td>FT B cells</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Splenocytes</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-4 + IL-5 + LPS</td>
<td>FT B cells</td>
<td>6.2</td>
<td>7.1</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Splenocytes</td>
<td>208.0</td>
<td>43.6</td>
<td>9.9</td>
</tr>
<tr>
<td>IL-4 + IL-5 + CD40LT</td>
<td>FT B cells</td>
<td>0.8</td>
<td>0.3</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Splenocytes</td>
<td>8.8</td>
<td>3.2</td>
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</tbody>
</table>

*Thy-1low, HSAhigh cells were purified by cell sorting from day 15 fetal thymic (FT) lobes cultured for 7 days in LSC supplemented with flt3L + IL-7. The Thy-1low, HSAhigh cells were used to repopulate dGuo-treated fetal thymic lobes. Thirteen days after repopulation, the lobes were harvested, a cell suspension was prepared, and the cells were cultured with either 1) medium, 2) IL-4 (10 ng/ml) + IL-5 (10 ng/ml) + LPS (25 μg/ml), or 3) IL-4 (10 ng/ml) + IL-5 (10 ng/ml) + CD40LT (3 μg/ml) at 10^5 cells/well. After 7 days the supernatants were collected, and the concentration of IgM, IgG1, and IgE was determined by ELISA. Unfractionated splenocytes derived from adult mice were cultured under the same stimuli for Ig production comparison. Data shown are representative of three separate experiments.

Thymocytes were isolated from day 15 fetal thymic lobes, and their proliferative response to media alone, LPS (25 μg/ml), soluble anti-IgM (10 μg/ml), and IL-4 (10 ng/ml) + IL-5 (10 ng/ml) + CD40LT (3 μg/ml) was measured (3). The proliferative response of freshly isolated splenocytes from adult C57BL/6 mice was included for comparison (2). Data presented are the mean ± SEM of triplicates and representative of three separate experiments.

The presence of progenitors in the fetal thymus and mature B cell potential, the irradiated adult thymus does not support their development (8). More recently, a cell with the phenotype B220^medium, slg^+, CD43^− was isolated from the adult thymus and, upon intrathymic transfer into nonirradiated neonatal thymus, was able to generate mature thymic B cells (9). In addition, the observations that progenitors from the adult thymus (10) and fetal liver (23) can develop into B cells when cultured on thymic stromal lines suggest that the stromal components of the thymus can support B cell development in vitro.
flt3L and IL-7 have a role in conventional B cell development. In in vitro studies, flt3L augments B cell commitment and development from bone marrow-derived progenitors when added to cytokines such as IL-7 and c-kit ligand (35–38). The implication that flt3L has an important role in B cell development is supported by studies on mice in which the flt3R is mutated, resulting in flt3R<sup>−/−</sup> mice. Reduced numbers of pro-B and pre-B cells were noted in the bone marrow (39). Blocking IL-7 activity in mice with an anti-IL-7 Ab had a dramatic effect on both B cell and T cell development (40), and mice lacking the IL-7R (IL-7R<sup>−/−</sup>) had a severe defect in both the B cell and T cell compartments (41). B cell development in the bone marrow was blocked at the pre-B cell stage, implying that a signal from IL-7 is required for those cells to develop into Ig-expressing B cells (40, 41). Our results described in this work suggest that, like the bone marrow-derived B cell progenitors, thymic progenitors with B cell potential are also responsive to the combination of flt3L + IL-7.

Effects of flt3L and IL-7 on T cell progenitors have also been described. One group that originally purified flt3L from a thymic stromal line (29) reported that flt3L alone and in combination with IL-7 induces proliferation of fetal thymocytes. The addition of flt3L to cultures of thymic stromal cells and IL-12 augmented T cell generation from bone marrow-derived CD34<sup>+</sup> cells (30). When primitive CD4<sup>low</sup> cells isolated from the adult thymus were cultured in a mixture of cytokines (IL-3 + IL-6 + IL-7), they maintained their ability to reenter a fetal thymic lobe and generate T cells. The addition of flt3L to the mixture augmented the expansion of these primitive cells such that T cell production was increased in the repopulated lobes (42). It was concluded that flt3L delivers an expansion signal to these primitive cells, rather than a differentiation signal (42). It appears that like IL-7, flt3L is involved in the regulation of early events in both T and B cell lymphopoiesis.

Thymic B cells have some unique properties when compared with peripheral B cells (17, 21, 22). The B cells we described in this work that developed in the fetal thymus share some features similar to those of adult thymic B cells. Similarities with adult thymic B cells included relatively low levels of B220 expression on the cell surface and the presence of CD5<sup>+</sup> and CD5<sup>−</sup> subsets of B cells (Fig. 5). CD5 expression on peripheral B cells is restricted to a very minor subset (20), but is present on both thymic and peritoneal cavity B cells. The thymic B cells did not proliferate in response to the B cell stimuli, LPS, or soluble anti-IgM (Fig. 7), as has been previously reported for adult-derived thymic B cells (21). However, thymic B cells do proliferate in response to CD40L + IL-10 (22), and we observed that IL-4 + IL-5 + CD40L stimulated proliferation of the fetal thymic B cells (Fig. 7). Although only a fraction of the thymic B cells developed to the stage in which they expressed cell IgM (up to 30%, after approximately 14 days intrathymically) (Fig. 5), they responded to the B cell stimuli of IL-4 + IL-5 + CD40L or IL-4 + IL-5 + LPS to release soluble IgM and undergo isotype switching to produce IgG1 and IgE (Table III). It is not clear whether intrathymic B cells produce soluble IgGs in the thymus, and if they do, what purpose they serve. One difference between adult thymic B cells and the fetal thymic B cells we describe in this work is the lack of CD11b expression on the fetal thymic B cells (Fig. 4B). Adult thymic B cells express CD11b (17), as do peritoneal CD5<sup>+</sup> B cells (20).

We conclude that the fetal thymus contains progenitors that have B cell potential. The combination of flt3L + IL-7 added to intact fetal thymic lobe cultures supported the survival of these B cell progenitors for 7 days in vitro. Upon reentry into an alymphoid fetal thymic lobe, B cell maturation to the stage of a functional IgM<sup>+</sup> cell occurred, demonstrating that the fetal thymic environ-


