Induction of T Cell Development from Hematopoietic Progenitor Cells by Delta-like-1 In Vitro

Thomas M. Schmitt and Juan Carlos Zúñiga-Pflücker
Department of Immunology
University of Toronto
Sunnybrook and Women’s College Health Sciences Centre
2075 Bayview Avenue
Toronto, Ontario M4N 3M5
Canada

Summary

The molecular interactions provided by the thymic microenvironment that predicate T cell development remain obscure. Here, we show that a bone marrow stromal cell line ectopically expressing the Notch ligand Delta-like-1 loses its ability to support B cell lymphopoiesis, but acquires the capacity to induce the differentiation of hematopoietic progenitors into CD4 CD8 double- and single-positive T cells. Both γδ-TCR+ and αβ-TCR+ T cells are generated, and CD8+ TCRαβ cells produce γ-interferon following CD3/TCR stimulation. These results establish that expression of Delta-like-1 on stromal cells provides key signals for the induction of T cell lineage commitment, stage-specific progenitor expansion, TCR gene rearrangement, and T cell differentiation in the absence of a thymus. Thus, it is likely that Delta-like-1/Notch interactions by the thymus underpin its unique ability to promote lineage commitment and differentiation of T cells.

Introduction

The development of the various hematopoietic cell lineages is compartmentalized during fetal development and throughout adult life. At approximately day 12 of embryonic development, the fetal liver (FL) is seeded by definitive hematopoietic stem cells, which arise from the aorta-gonad-mesonephros region of the developing embryo (Cumano and Godin, 2001). The FL continues as the primary site of hematopoietic development until birth, when the bone marrow (BM) takes over as the primary site for hematopoiesis in the adult.

The process of stem cell differentiation is tightly regulated by soluble factors and cell contact-dependent signals within specialized microenvironments, each of which supports the development of specific cell lineages. During lymphopoiesis, the thymic environment is required for the differentiation of hematopoietic progenitor cells (HPCs) into T lymphocytes (Anderson et al., 1996). On the other hand, B cell development takes place within the BM microenvironment (Osmond, 1994).

The process of hematopoiesis can be modeled in vitro using BM-derived stromal cell lines (Dorschkind, 1990). A number of BM-derived stromal cell lines have been developed in recent years that are capable of supporting the development of multiple hematopoietic cell lineages. For instance, the OP9 BM stromal cell line (Kodama et al., 1994) has been shown to support the differentiation of HPCs into multiple lineages, including B cells, in vitro (Carlyle et al., 1997). However, efforts to generate T cells from HPCs in vitro have been unsuccessful. This is thought to be due to unknown factors acting at multiple developmental stages within the three-dimensional architecture of the thymus (Anderson et al., 1996; Lind et al., 2001).

The process of T cell development from HPC to mature TCRαβ+ T cell consists of a series of commitment events and multiple developmental checkpoints, including TCR V(D)J gene rearrangement, TCR-β-selection, and positive/negative selection of developing thymocytes. Efforts to recapitulate one or more of these events in vitro have had to depend on the use of fetal thymic organ culture (Anderson et al., 1996). The molecular interactions responsible for this thymus dependency remain largely unknown. However, a number of recent studies have implicated Notch receptor-ligand interactions in the earliest T cell lineage commitment events (MacDonald et al., 2001). Notch signaling is an evolutionarily conserved pathway that controls multiple cell fate decisions throughout ontogeny. Notch signaling is initiated by the local interaction of Notch receptors with Notch ligands on neighboring cells (Artavanis-Tsakonas et al., 1999). In vertebrates, these ligands consist of Jagged and Delta-like family members. Engagement of the Notch receptor results in its proteolytic cleavage by a presenilin-dependent γ-secretase activity (Taniguchi et al., 2002), followed by the translocation of the cleaved intracellular domain of Notch to the nucleus, where it binds to CBF-1/RBP-Jκ and activates transcription of downstream target genes (Artavanis-Tsakonas et al., 1999).

Several lines of evidence implicate Notch signaling at various stages of lymphocyte development. Specifically, it has been suggested that Notch signaling promotes TCRαβ+ T cell development at the expense of TCRγδ+ T cell development (Washburn et al., 1997). Furthermore, a number of investigators have proposed various roles for Notch in the development of CD4+ and CD8+ single-positive (SP) T cells from CD4-CD8- (DP) precursor thymocytes (Deftos et al., 2000; Izon et al., 2001; Robey et al., 1996; Wolfer et al., 2001). However, the most striking data come from studies that address the role of Notch signaling in governing T cell versus B cell fate decisions by lymphocyte progenitors. Specifically, B cell development is abolished in mice reconstituted with BM progenitors expressing a constitutively active form of Notch; rather, DP T cells develop in the BM of these mice (Pui et al., 1999). In a complementary experiment, Notch-1 conditionally deficient mice show a severe block in T cell development, with the concomitant development of B cells in the thymus (Radtke et al., 1999). These results strongly support the notion that Notch signaling is critical for the earliest stages of T cell commitment. A further role of Notch signals at early stages of lymphocyte development would be the induction of cell fate decisions in the BM and the thymus.
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stages of T cell development has recently begun to be elucidated. Radtke and colleagues demonstrated that a conditional inactivation of Notch-1 at the CD44⁺CD25⁻ (DN2) stage of T cell development results in a partial block at the subsequent CD44⁺CD25⁺ (DN3) stage (Wolfer et al., 2002). This was shown to be the result of inefficient V to DJ recombination at the TCR-γ locus. In contrast, rearrangements at the TCR-γδ locus were not affected. Taken together, these findings support the notion that Notch signaling plays a critical role in T cell lineage commitment.

In light of these findings, we hypothesized that BM stromal cell lines, which have been extensively used for the differentiation of B lymphocytes from HPCs, may fail to express specific Notch ligands necessary for T cell commitment and differentiation. Thus, ectopic expression of an appropriate Notch ligand by a BM stromal cell line might induce differentiating HPCs to adopt a T cell fate by redirecting commitment away from the B cell lineage. In an effort to generate an in vitro system for the induction of T cell lineage commitment by FL-derived HPCs, OP9 BM stromal cells were retrovirally transduced to express the Notch ligand Delta-like-1. The resulting Delta-like-1-expressing OP9 cell line (OP9-DL1) lost the ability to support B cell lymphopoiesis, while gaining the capacity to induce a normal program of T cell differentiation from FL-derived HPCs, including the generation of DP and SP T cells. Strikingly, HPCs induced to differentiate on OP9-DL1 cells underwent robust cellular expansion and gave rise to both γδ⁺TCR⁺ and αβ⁻TCR⁻ T cells. Moreover, CD8 SP TCR⁺ cells produced γ-interferon following CD3/TCR stimulation, demonstrating that functionally mature T cells were generated. These results establish that ectopic expression of Delta-like-1 on OP9 BM stroma cells provides the necessary signals that are responsible for the induction of T cell lineage commitment, TCR V(D)J rearrangement, and T cell differentiation by HPCs in the absence of a thymus.

Results

Expression of Notch Ligands by OP9 Cells

Although OP9 cells have been shown to support the differentiation of HPCs into multiple lineages (Kodama et al., 1994), including B cells (Carlyle et al., 1997), efforts to induce T cell differentiation in vitro have been unsuccessful in the absence of a thymic microenvironment. To determine whether OP9 cells fail to express Notch ligands, which may provide essential cell contact-dependent signals required for T cell commitment and differentiation (Radtke et al., 1999), we analyzed OP9 cells for the expression of these molecules by reverse transcriptase-PCR (Figure 1). This analysis revealed that transcripts for Delta-like-1 and Delta-like-4 were undetectable in OP9 cells, while these transcripts were present in thymus stroma-enriched cell suspensions (Figure 1). On the other hand, transcripts for Jagged-1 and Jagged-2 were detected in both OP9 cells and thymic stroma cells (Figure 1).

Delta-like-1 has been shown to efficiently engage and induce Notch receptor signaling (Kuroda et al., 1999). We reasoned that the lack of Delta-like-1 expression by OP9 cells may be responsible for their inability to sup-
Induction of T Cell Development by Delta-like-1

Figure 4. Differentiation and Proliferation of Pre-B Cells Cultured on OP9 Cells or OP9-DL1 Cells
Flow cytometric analysis for surface expression of CD19 and CD117 of pre-B cells (CD117⁺ B220⁻ CD19⁻) sorted from the adult BM and cultured for 7 days on either control OP9 cells or OP9-DL1 cells.

Figure 3. Lineage Commitment and Differentiation of HPCs Cultured on OP9-GFP Cells or OP9-DL1 Cells
Flow cytometric analysis for B cell and NK cell markers (CD19 and NK1.1, respectively) or T cell markers (CD4 and CD8) from HPCs pre-cultured on either OP9-GFP cells or OP9-DL1 cells for 4 days (A), 7 days (B), or 12 days (C). The difference in the levels of NK1.1 staining in the left panels of (B) and (C) is due to the use of different fluorochrome-labeled anti-NK1.1 mAbs.

placed in culture either with OP9-GFP cells or OP9-DL1 cells. Flow cytometric analysis was performed at several time points during the coculture period to determine the ability of each stromal cell line to support the differentiation of HPCs into various lymphoid lineages (Figure 3). As expected, HPCs cocultured with OP9-GFP cells did not give rise to T cells, and therefore these cultures did not contain CD4- or CD8-expressing cells (Figures 3A–3C). Rather, as previously described (Carlyle et al., 1997), HPCs cultured with OP9-GFP cells gave rise to B cells and NK cells within 4 days, and these cells expanded throughout the culture period (Figures 3A–3C). In striking contrast, HPCs cultured on OP9-DL1 cells gave rise to CD4⁺ CD8⁻ immature DP T cells after 7 days of coculture, and these DP T cells accounted for the majority of HPC-derived cells by day 12 (Figures 3B–3C). The temporal kinetics of DP T cell differentiation in the presence of OP9-DL1 cells is similar to that observed following transfer of HPCs into fetal thymic organ culture (Carlyle et al., 1997). We also observed approximately a 100-fold increase in cellularity during the first week of culture, with the cell yields continuing to increase another 15- to 20-fold by day 12. In addition to CD4- and CD8-expressing cells, day 12 cultures also contained a small population of presumably mature SP T cells (see below). Both DP and CD8 SP T cells derived from HPC/OP9-DL1 coculture expressed CD8β as well as CD8α on the cell surface (data not shown).

Notably, HPCs cocultured with OP9-DL1 cells failed to give rise to B cells, while the differentiation of NK cells was readily observed throughout the coculture period (Figures 3A–3C). These findings are consistent with the interpretation that Notch/Delta-like-1 interactions induce the commitment and differentiation of FL-derived HPCs toward the T cell lineage, while inhibiting the development of B cells.

OP9-DL1 Cells Support the Growth of Committed Pre-B Cells
To determine whether the absence of B cells among the progeny of HPCs cultured on OP9-DL1 cells resulted from an inhibition of B cell commitment or growth, we isolated committed CD117⁺ CD45R/B220⁺ CD19⁻ pre-B cells from adult BM (ten Boekel et al., 1997). These pre-B cells were placed in culture with either OP9 or OP9-DL1 cells for 7 days and then analyzed by flow cytometry. Figure 4 shows that pre-B cells cultured on either OP9 cells or OP9-DL1 cells were able to proliferate and further differentiate into the more mature CD117⁻ CD19⁺ stage. After 7 days, a small percentage of cells from each culture also expressed surface IgM (data not shown). In keeping with the pre-B cell phenotype of the starting population, neither T cells nor NK cells were detected in these cultures. Since pre-B cells have been
Table 1. Progenitor Frequency Analysis for HPCs Cultured on OP9-GFP or OP9-DL1 Cells

<table>
<thead>
<tr>
<th>Lineage Analyzed</th>
<th>Progenitor Frequency (^1) (95% confidence limits)(^2)</th>
</tr>
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<tbody>
<tr>
<td>T cells(^3)</td>
<td>17 (12.6–22.7)</td>
</tr>
<tr>
<td>B cells(^3)</td>
<td>6 (4.7–8.4)</td>
</tr>
<tr>
<td>Myeloid cells(^3)</td>
<td>5 (3.5–6.3)</td>
</tr>
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\(^1\) Individual wells (n = 36) were analyzed for the generation of T cells (CD4\(^+,\) CD8\(^+\)); B cells (CD19\(^+\)); and myeloid cells (CD11b\(^+\)/Mac-1\(^+\)) (Supplemental Figure S1 [http://www.immunity.com/cgi/content/full/17/6/749/DC1]).

\(^2\) Statistical analysis was performed using the method of maximum likelihood applied to the Poisson model.

\(^3\) T cells were generated by culturing HPCs on OP9-DL1 cells. B cells and myeloid cells were generated by culturing HPCs on OP9-GFP cells.

shown to express Notch-1 (Bertrand et al., 2000), these data suggest that the Delta-like-1-mediated inhibition of B lineage development observed in Figure 3 occurs at the earliest stages of lymphocyte lineage commitment, as it does not affect already-committed B cell precursors.

Efficient Generation of T Cells from FL-Derived HPCs Cultured on OP9-DL1 Cells

To determine the frequency of progenitors present within the FL-derived HPC population that, when cultured on OP9-DL1 cells, could give rise to T cells, a limiting dilution analysis was performed. We also determined the progenitor frequency for B cells and myeloid cells from HPCs cultured on control OP9 cells. Table 1 shows the progenitor frequencies obtained from 1, 3, 10, or 30 HPCs (n = 36 per group) cultured on OP9-GFP or OP9-DL1 cells for 12 days and then analyzed by flow cytometry for the presence of B and myeloid cells or T cells, respectively (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/17/6/749/DC1). The presence of DP T cells was used as evidence for T cell progenitor potential within individually harvested wells (Supplemental Figure S1). The progenitor frequency of HPCs that can give rise to T cells was determined by the method of maximum likelihood and calculated to be about 1 in 17 HPCs. This progenitor frequency is inconsistent with the possibility that the observed T cells were derived from an extremely rare population of T cell lineage-committed pro-T cells that may exist within the HPC fraction (Kawamoto et al., 1999). The observed T cell progenitor frequency from HPCs was ~3-fold lower than that obtained for B cells, which was determined to be about 1 in 6 HPCs (Table 1). The myeloid progenitor frequency was about 1 in 5 HPCs, which resembles the frequency of true multipotent hematopoietic progenitor cells observed by others within the FL CD117\(^+\) Sca-1\(^+\) fraction (Kawamoto et al., 1999). Taken together, these results indicate that T cell lineage commitment occurs with high efficiency from FL-derived HPCs cultured on OP9-DL1 cells.

OP9-DL1 Cells Support a Normal Program of T Cell Development

Intrathymic T cell development proceeds through successive steps that are characterized by the differential surface expression of CD44 and CD25 within the CD4\(^-\) CD8\(^-\) population (Shortman and Wu, 1996). To address whether T cells derived from HPCs cultured on OP9-DL1 cells followed the normal pattern of development observed in the thymus, cells from day 7 cocultures, which mostly display a CD4\(^-\) CD8\(^-\) surface phenotype (Figure 3B), were analyzed for CD44 and CD25 surface expression (Figure 5A). Strikingly, these cells exhibited a pattern of CD44/CD25 expression that is similar to that observed among immature CD4\(^-\) CD8\(^-\) thymocytes. The similarities between T cell development occurring in the thymus and on the OP9-DL1 cells also applied to the regulation of DNA rearrangement at the TCR-\(\beta\) locus. In this regard, a similar pattern of D\(_{\beta}\)-J\(_{\beta}\) rearrangement was observed from DNA obtained either from the day 7 coculture cells (total or sorted CD44\(^-\) CD8\(^-\) cells) or from day 14 fetal thymocytes (Figure 5A). In contrast, and as expected, DNA from fibroblasts was in germline...
configuration at the same locus. These results indicate that T lineage cells that develop from HPC/OP9-DL1 cocultures undergo a normal program of differentiation.

Two distinct lineages of TCR-bearing T cells normally develop in the thymus (Shortman and Wu, 1996). To determine whether both lineages could be generated from HPCs cultured on OP9-DL1 cells, we analyzed cells from day 12 cocultures for $\alpha\beta$- and $\gamma\delta$-TCR surface expression (Figure 5B). As expected, HPCs cultured on OP9 cells did not contain $\alpha\beta$- or $\gamma\delta$-TCR-bearing cells after 12 days in culture. In contrast, HPCs cocultured with OP9-DL1 cells contained both $\alpha\beta$- and $\gamma\delta$-T cells. Furthermore, to determine whether a broad distribution of TCR-V$\beta$ usage occurred during T cell differentiation on OP9-DL1 cells, we analyzed for the expression of several V$\beta$ chains commonly used by T cells derived from Swiss.NIH mice, which are of the V$\beta_2$ haplotype. Figure 5C shows that multiple V$\beta$ chains (V$\beta$-3, -6, and -17a) are expressed, with no apparent bias in V$\beta$ usage. Furthermore, TCR-V$\beta$ chains were clonally expressed (data not shown), indicating that allelic exclusion at the TCR$\beta$ gene locus was enforced.

**Generation of Functionally Mature T Cells on OP9-DL1 Cells**

Although the majority of T lineage cells generated from HPC/OP9-DL1 cocultures corresponded to immature DP T cells, a small percentage of SP T cells were present in day 12 cocultures (Figure 3C) and at later time points (Figure 6A). In order to determine whether these cells represented DPs that had differentiated to the next stage of T cell development, SP T cells present in day 17 HPC/OP9-DL1 cocultures were compared to thymocytes obtained from a 2-week-old mouse (Figure 6A). Figure 6B shows that ~16% of the CD8 SP T cells from these cocultures expressed surface TCR levels similar to those observed on CD8 SP thymocytes. On the other hand, CD4 SP cells obtained from these cocultures did not express surface TCR at levels that were similar to those observed on CD4 SP thymocytes (Figure 6B).

The observation that some CD8 SP T cells expressed high levels of TCR on their surface suggested that these cells might have reached functional maturity. To address this intriguing possibility, CD4$^+$ CD8$^+$ TCR$^{hi}$ cells were isolated by flow cytometric cell sorting, and their response to plate-bound antibodies specific for CD3 and CD28 was determined (Figure 6C). The stimulated CD8$^+$ T cells but not the unstimulated controls underwent a burst of activation-induced proliferation and $\gamma$-interferon production, as detected by flow cytometric analysis (Figure 6C). These data demonstrate that expression of Delta-like-1 by OP9 cells is able to support the differentiation of FL-derived HPCs into mature and functional T cells in vitro.

**Discussion**

Notch is responsible for multiple binary cell fate decisions in the developing embryo, and Notch signals regulate both proliferative and apoptotic events in a cell-context-dependent manner (Artavanis-Tsakonas et al., 1999). Notch receptor/ligand interactions have been implicated in governing the commitment of common
lymphoid progenitors (CLPs) to the T cell lineage at the expense of B lineage development (Koch et al., 2001; Pui et al., 1999; Radtke et al., 1999). Consistent with these findings, we show that OP9 cells expressing Delta-like-1 induce the commitment of FL-derived HPCs toward the T cell lineage while subverting their ability to support B cell lymphopoiesis. This likely occurs at a CLP-like intermediate stage during the coculture period. The fact that NK cells develop from HPCs regardless of the expression of Delta-like-1 by OP9 cells suggests that commitment of CLPs to the NK cell lineage is not mediated by the Notch-mediated T/B cell fate determination.

Our results demonstrate that FL-derived HPCs differentiate into T cells with a high efficiency (1 in 17 HPCs) when cultured on Delta-like-1-expressing OP9 cells. This high progenitor frequency supports the notion that the resulting T cells are derived from multipotent progenitors or CLPs induced to adopt a T cell lineage fate, rather than from a rare subset of precommitted T cell progenitors that might be present within the HPC fraction (Kawamoto et al., 1999). Moreover, not only did T cell lineage commitment occur at a high frequency, but it was also accompanied by high cellular yields due to the robust proliferative potential of the differentiating HPCs. This is highlighted by the fact that after 12 days of coculture, a cellular expansion of $\approx 2,000$-fold was routinely observed. Interestingly, despite the fact that the B cell progenitor frequency of HPCs was 3-fold higher (1 in 6 HPCs), the cellular expansion observed for T lineage cells generated from HPCs cultured on OP9-DL1 cells was about 3-fold higher than that of B cells obtained from OP9-GFP cocultures.

Our findings suggest that the differentiation requirements for B cell and T cell lymphopoiesis are remarkably similar. This is illustrated by the fact that the expression of one additional molecule, Delta-like-1, switches the ability of a stromal environment that otherwise strongly supports B cell differentiation to now induce and support the full development of T cells from FL HPCs, as well as from adult bone marrow-derived HPCs (data not shown). A similar approach was recently described by Jaleco et al., in which a different bone marrow stromal cell line, S17 (Collins and Dorshkind, 1987), was transduced to express Delta-like-1 (Jaleco et al., 2001). Consistent with our findings, S17 cells expressing Delta-like-1 lost the ability to induce B lymphopoiesis from human-derived CD34$^+$ hematopoietic progenitors. However, Delta-like-1-expressing S17 cells only promoted the emergence of a cell population that resembled T/NK progenitors. In contrast to our results using OP9-DL1 cells, no clear evidence for T cell lineage commitment or further T cell development was reported (Jaleco et al., 2001). Several factors could explain the observed differences: human progenitors may be unable to differentiate into T cells on a mouse stromal cell line; or, on the other hand, S17 cells may not provide the appropriate microenvironment for efficient Delta-like-1-mediated T cell development. To address the latter possibility, we generated S17 cells expressing Delta-like-1 (S17-DL1) and compared them to OP9-DL1 cells for the ability to induce and support T cell development from HPCs (see Supplemental Figure S2 at http://www.immunity.com/cgi/content/full/17/6/749/DC1). Although B cells were generated with equal efficiency from HPCs cultured on control OP9-GFP or S17-GFP cells, there was a marked difference in the generation of T cells on S17-DL1 cells ($\approx 5\%$ DP cells) as compared to OP9-DL1 cells ($\approx 65\%$ DP cells). Furthermore, HPCs cultured on S17-DL1 cells displayed little proliferative potential during the culture period, similar to the limited growth potential observed with human progenitors cultured on S17 cells expressing Delta-like-1 (Jaleco et al., 2001).

Our cocultures include the addition of exogenous cytokines (Flt3L and IL-7), which may account for the moderate generation of T cells on S17-DL1 cells in our hands (Supplemental Figure S2), as opposed to the cocultures with human CD34$^+$ cells, which did not receive exogenous cytokines (Jaleco et al., 2001). Therefore, OP9 cells provide a more appropriate stromal environment for Delta-like-1 to mediate the induction of T cell lineage commitment and differentiation than S17 cells, which may lack important factors and/or express negative modulators of Notch receptor or Notch ligand function, such as Fringe or Neuralized, respectively (Artavanis-Tsakonas et al., 1999; Justice and Jan, 2002; Koch et al., 2001). Thus, our data showing the inefficient ability of S17-DL1 cells to induce T cell development, together with the use of human progenitors on a mouse stromal cell line by Jaleco et al. (2001), serve to clarify the apparent differences between the previously reported observations and the findings presented here.

An important implication of our data is that the three-dimensional thymic microenvironment is not dispensable for T cell development (Anderson et al., 1996; Carlyle and Züniga-Pflücker, 1998; Lind et al., 2001). Lineage commitment, TCR gene rearrangement, and progression to the DP stage following pre-TCR formation can be recapitulated in vitro by HPCs cultured on OP9 cells expressing Delta-like-1. In this regard, part of the observed burst of cellular proliferation appears to take place just prior to the appearance of DP cells, coinciding with the time TCR$\beta$ selection normally occurs (Kruisbeek et al., 2000). In support of this notion, OP9-DL1 cocultures using HPCs derived from recombinase-activating gene (RAG)-2-deficient mice (Shinkai et al., 1992), which are unable to generate a TCR$\beta$ chain, failed to reach the DP stage of T cell development and displayed the expected block in T cell differentiation at the CD44$^+$CD25$^-$ stage (data not shown). Thus, HPCs differentiating on OP9-DL1 cells appear to follow a normal program of early T cell development, as the observed stage-specific expansion and progression to the DP stage appear to be dependent on the induction of TCR$\beta$ rearrangement and the resulting $\beta$-selection-mediated signaling outcomes.

The development of $\gamma\delta$-T cells on OP9-DL1 cells demonstrates that both $\gamma\delta$- and $\alpha\beta$-lineages can develop in the presence of Delta-like-1, suggesting that Notch signals mediated by Delta-like-1 do not influence commitment to the $\gamma\delta$- or $\alpha\beta$-T cell fate (Washburn et al., 1997). Although a number of studies have focused on the role of Notch signaling in CD4 versus CD8 lineage development (Deftos et al., 1998, 2000; Izon et al., 2001; Robey et al., 1996; Wolfer et al., 2001), the presence of mature TCR$\alpha\beta$ CD8$^+$ SPs but not CD4$^+$ SP T cells most likely reflects the fact that OP9 cells express MHC class I but do not express MHC class II. In keeping with this,
we frequently observed the appearance of CD4+ CD8+ TCR+ cells, which are consistent with cells undergoing the first stages of CD4- or CD8-lineage commitment (Brugnera et al., 2000), while the few CD4- CD8- SP cells express TCR levels that are below those of normal CD4 SP thymocytes and therefore likely represent developmental intermediates. In contrast, a small but significant number of CD4+ CD8+ TCR+ cells are generated, and, when isolated, these are capable of responding to TCR stimulation by producing γ-IFN, demonstrating that mature SP cells can be generated from HPCs induced to differentiate on Delta-like-1-expressing OP9 cells.

In addition to the previous reports addressing the role of Notch receptor/ligand interactions at various stages of lymphocyte development (Deftso et al., 1996; 2000; Izon et al., 2001; Koch et al., 2001; Pui et al., 1999; Radtke et al., 1999; Robey et al., 1996; Wolfer et al., 2001), we now demonstrate that Delta-like-1-induced signals are responsible for the commitment and differentiation of genetically unmanipulated HPCs into T cells in the absence of a thymic environment. These findings should simplify the experimental approaches heretofore required for the study of T cell differentiation, and may enable the development of immune-reconstitution approaches employing defined sources of stem cells.

**Experimental Procedures**

**Mice**

Timed-pregnant Swiss NIH mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). RAG-2-deficient mice (Shinkai et al., 1992) were bred and maintained in our animal facility.

**Flow Cytometry and Cell Sorting**

Flow cytometry was performed using a FACScalibur (BD Biosciences, San Diego, CA) instrument, as previously described (Carlyle and Zuñiga-Pflücker, 1998) and then sorted for expression of CD117 and Sca-1. Sorted cells were placed in culture for 12 days, after which cells were harvested and counted Trypan blue-excluding cells. Wells that were determined by postsort analysis. clone size.

**OP9-DL1 Cells**

OP9 cells (Kodama et al., 1994) were infected with the empty MigR1 retroviral vector (Pui et al., 1999) or with the MigR1 retroviral vector engineered to express the Delta-like-1 gene (Kuroda et al., 1999)’s of the internal-ribosomal entry site, allowing the bicistronic expression of Delta-like-1 and green fluorescent protein (GFP). The MigR1 retroviral backbone was obtained from W. Pear (University of Pennsylvania, PA). The retroviral vectors were packaged using the PT67 retroviral packaging cell line (Clonect-BD Biosciences). The Delta-like-1 gene containing a 3’ T7 tag was kindly provided by T. Honjo (Kyoto University, Japan). Retrovirally transduced OP9 cells were sorted on the basis of GFP expression, and expression of the Delta-like-1:T7 product was determined by intracellular staining (see Figure 2).

**Hematopoietic Progenitor Cell and OP9 Cell Cocultures**

Hematopoietic progenitor cells were isolated from day 14 FL cells. CD24+/Lin- FL progenitor cells were enriched by anti-CD24 antibody/-complement-mediated depletion (Carlyle and Zuñiga-Pflücker, 1998) and then sorted for expression of CD117 and Sca-1. Sorted CD117+ Sca-1+ HPCs were seeded at 4 x 10^5 cells/well into 24-well tissue culture plates containing a confluent monolayer of OP9-GFP cells or OP9-DL1 cells. Pre-B cells were sorted from adult BM for CD117+ CD45R+ CD19+ cells and seeded at 4 x 10^5 cells/well into 24-well tissue culture plates containing a confluent monolayer of OP9 cells or OP9-DL1 cells. All cocultures were performed in the presence of 5 ng/ml IL-7 and 5 ng/ml Flt3L (Peprotech, Rocky Hill, NJ). Cocultures were harvested by forceful pipetting at the indicated time points.

**PCR and RT-PCR**

Genomic DNA was purified from embryonic fibroblasts, d14 fetal thymus, cells obtained from day 7 HSC/OP9-DL1 cocultures, and cocultured cells sorted for a CD4+ CD8+ phenotype using the EasyDNA kit (Invitrogen, Carlsbad, CA). 100 ng of each DNA sample was amplified using a PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, MA). Primers used for the TCR Dβ-Jβ rearrangement analysis have been previously described (Rodewald et al., 1994). Products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. All PCR products shown correspond to expected molecular sizes. For RT-PCR analysis, single-cell suspensions were prepared from OP9 cells and from d14 fetal thymuses treated for 7 days in organ culture with 1.1 mM deoxyguanosine. Isolation of total RNA and RT reactions was carried out as previously described (Carlyle and Zuñiga-Pflücker, 1998). All semiquantitative PCR reactions were performed using the same serially diluted cDNA batches as shown for β-actin. Gene-specific primers used for PCR are as follows: Notch-1 upper GAGCCGGTGTAAGGTAGT, lower AATCCTGGTGGGGAATG; TdT upper TCTCAGCAGCCTGCCATAAC, lower TTTTACAGGGGTTGCTCTCG; Jagged-1 upper TTGAAAGCCGTGTTGAAAC, lower TAAATAGCCTGCAATCCGT; Delta-like-1:ac (ac) GTACCTGCGGTGGGGGAATGTC; Jagged-1 upper TCTCTGACCCTGCCATAAC, lower TTTTACAGGGGTTGCTCTCG; Jagged-2 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper AACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4 upper GAAAGAGCCGGTGTGAAA, lower TAAATAGCCCGCCAATCGGT; Delta-like-4 upper ACCTCGGGATGACGCCTTTG, lower AGACCAC CACAGACGACAG; and Delta-like-4.
References


