Direct Comparison ofDll1- and Dll4-Mediated Notch Activation Levels Shows Differential Lymphomyeloid Lineage Commitment Outcomes

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Discriminated according to surface expression of CD44 and CD25 as they lack both CD4 and CD8 expression, but can be further mature thymocytes are referred to as double negative (DN) cells, the detection of cell surface markers (7, 8). Briefly, the most important lineage differentiation steps that are typically monitored through expressed on thymic epithelial cells (TECs), inducing a series of T cells (HPCs), which seed the thymus, interacts with the ligand Dll4 (3–6). The receptor Notch1 expressed on hematopoietic progenitor cells (HPCs), which seed the thymus, interacts with the ligand Dll4 expressed on thymic epithelial cells (TECs), inducing a series of T lineage differentiation steps that are typically monitored through the detection of cell surface markers (7, 8). Briefly, the most immature thymocytes are referred to as double negative (DN) cells, as they lack both CD4 and CD8 expression, but can be further discriminated according to surface expression of CD44 and CD25.

In the thymus, Notch signaling is essential for T lymphopoiesis, with Delta-like (Dll)4 uniquely involved in this process. However, using cocultures, either Dll4 or Dll1 were shown to support T lymphopoiesis. To address which Dll is more effective at inducing hematopoietic progenitor cells to give rise to T lineage cells in vitro, we generated OP9 cells expressing a series of incrementally discrete and equivalent levels of Dll1 or Dll4. In keeping with previous findings, OP9 cells expressing high levels of either Dll1 or Dll4 gave rise to T lineage cells with similar efficacy, and prevented the differentiation of B and myeloid-lineage cells. However, at limiting levels, Dll4 maintained its ability to inhibit B lineage choice and induce T lineage commitment and differentiation at lower levels than Dll1. This manifest property of Dll4 is evident despite lower levels of steady-state surface expression than Dll1 on OP9 cells. The heightened effectiveness of Dll4 over Dll1 also corresponded to the induction of Notch target genes, and inhibition of B and myeloid-specific transcription factors. Furthermore, we show that OP9 cells expressing levels of Dll4 equivalent to those present in thymic epithelial cells, as expected, gave rise to T lineage cells, but were also permissive for the differentiation of myeloid cells; whereas, still inhibiting B lymphopoiesis. Our findings show that Dll4 expressed at physiological levels on OP9 cells is functionally distinct from similarly expressed levels of Dll1, illustrating the unique properties of Dll4 in supporting the combined T lineage and specific myeloid-lineage outcomes that underpin its function within the thymus. The Journal of Immunology, 2010, 185: 000–000.

The role of Notch receptor–ligand interactions in determining cell lineage fate choices at multiple developmental processes is well documented (1, 2). In the thymus, during T lymphopoiesis, these interactions are principally, and likely exclusively, mediated by Notch1 and Delta-like (Dll)4 (3–6). The receptor Notch1 expressed on hematopoietic progenitor cells (HPCs), which seed the thymus, interacts with the ligand Dll4 expressed on thymic epithelial cells (TECs), inducing a series of T lineage differentiation steps that are typically monitored through the detection of cell surface markers (7, 8). Briefly, the most immature thymocytes are referred to as double negative (DN) cells, as they lack both CD4 and CD8 expression, but can be further discriminated according to surface expression of CD44 and CD25.

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lineage differentiation outcomes. Lastly, our findings revealed that OP9 cells expressing Dll4 levels similar to those expressed by TECs allowed for the generation of both T lineage and specific myeloid cells, although still inhibiting B cell development, illustrating an important property of Dll4 that likely reflects its function within the thymus.

Materials and Methods

Mice

CD1 time-pregnant mice were obtained from Charles River Laboratories (Montreal, Quebec, Canada). Male C57BL/6 mice at 4–8 wk of age were used for BrdU assays. All experimental procedures related to the use of mice were approved and performed according to the guidelines specified by Sunnybrook Health Sciences Centre Animal Care Committee or by The Scripps Research Institute Animal Committee.

HPCs and OP9 cocultures

HPCs were obtained from fetal livers from timed-pregnant (embryonic day 15) CD1 mice that were depleted of mature lineage cells using anti-CD24 mAb (J11d.2; BD Biosciences, Mississauga, Ontario, Canada). Viable HPCs were subsequently sorted by flow cytometry as CD117+ Sca-1+ cells. The 1 × 10^7 HPCs were then seeded onto near confluent OP9 cell lines (as indicated) in 6-well plates in coculture media (α-MEM media [Invitrogen, Carlsbad, CA], 15% FBS [Thermo Fisher Scientific, Ottawa, Ontario, Canada], antibiotics penicillin [100 μg/ml] /streptomycin [100 U/ml] [Invitrogen], and cytokines Flt3-ligand [1 ng/ml; PeproTech, Rocky Hill, NJ] and IL-7 [1 ng/ml; PeproTech]) (15). Cells were harvested on day 6 for analysis or further passed onto fresh plates containing OP9 cells and coculture media for analysis on days 9 and 12.

TECs

TECs were isolated by first dispersing fetal thymuses from E16 embryos. Thymuses were trypsin and collagenase treated for 20 min at 37°C, mechanically sheared then subsequently immunodepleted of CD45-expressing cells using CD45-PE–conjugated anti-human (Jackson, Canada) via magnetic-assisted cell sorting. If needed, enrichment for epithelial cell adhesion molecule (EpCAM)-expressing cells was performed using anti-EpCAM-biotin (G8.8 mAb, developed by Dr. Farr and obtained from The Developmental Studies Hybridoma Bank developed under the auspices of the National Institutes of Children’s Health and Disease and maintained by The University of Iowa, Department of Biology, Iowa City, IA), after CD45-expressing cells were removed. Anti-EpCAM Ab was produced and conjugated to biotin in Sunnybrook Research Institute. MACS-LD columns (Miltenyi Biotec, Auburn, CA) were used to enrich EpCAM+ TECs, yielding a cell purity of 99% used for Western blot or gene transcription expression analysis. TECs were then used for various assays.

Flow cytometry cell sorting and analysis

For the detection of cell surface molecules, the following directly conjugated Abs were used: CD4-PE, CD8–allophycocyanin, CD11c–FITC, CD19–biotin, CD25–FITC, CD44–PE-Cy5, CD45–APC-Cy7, streptavidin–PE-Cy5, Dll4 mAb (kindly provided by Dr. Minghong Yan at Genentech, San Francisco, CA) and streptavidin-allophycocyanin was used to indirectly detect anti–EpCAM-biotin (G8.8 mAb, developed by Dr. Farr and obtained from the Developmental Studies Hybridoma Bank) or anti–EpCAM-biotin (G8.8 mAb, obtained from the Scripps Research Institute Animal Committee). For E16 thymic stromal cells enriched by CD45-PE exclusion columns, anti-MHC class II (I-A/I-E)-PE (BD Biosciences, Mississauga, Ontario, Canada). For E15 fetal liver-derived HPCs were cocultured with OP9-GFP or the various OP9-DLL1(Dll1)–/− cells (as indicated) for 48 h. Cells were then harvested and sorted by flow cytometry for cells expressing CD45. Total RNA was extracted with TRIzol reagent according to manufacturer’s instructions (Invitrogen). First-strand cDNA synthesis and PCR were performed using Reverse Transcripase III (Invitrogen) according manufacturer’s instructions. qRT-PCR was performed in triplicates on cDNA transcripts as amplified with SYBR Green (Invitrogen) containing poly- merase using a 7500 Real Time PCR System (Applied Biosystems) for 40 cycles. The results were analyzed using the Sequence Detection Software Version 1.4 (Applied Biosystems). Primer sequences used in qRT-PCR are provided in Supplemental Table I. Western blot analysis

Cells were lysed by treatment with ice-cold RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% deoxychylcine, 1% NP-40, 0.1% SDS) plus protease inhibitor (0.1 μM PMSF [Sigma-Aldrich] and Complete Mini protease inhibitor mixture [Roche, Laval, Quebec, Canada]). Protein quantifica- tion assay was done using BCA assay (Thermo Fisher Scientific, USA). SDS loading buffer (5×: 250 mM TrisHCl pH 6.8, 10% SDS, 30% Glycerol, 0.5M DTT, 0.02% bromophenol blue [all chemicals from Sigma-Aldrich]) was added to equivalent amounts of protein lysates from each sample. Proteins in the samples were electrophoretically separated and transferred onto polyvinylidene difluore membrane blotting membranes (Millipore, Billerica, MA). Membranes were blocked by 5% reconstituted milk overnight at 4°C, followed by incubation of ~1 h with primary Abs: rat-anti-HA (Sigma-Aldrich), mouse-anti–β-tubulin mAb (E7, developed by Michael Klymowski, sky, obtained from Developmental Studies Hybridoma Bank), or goat-anti- DLL4 (Abcam, Cambridge, UK) and streptavidin-HRP (Santa Cruz Biotechnology). Membranes were exposed to X-ray film and bands were visualized with enhanced chemiluminescence (ECL Western blotting Substrate, Pierce).
Thermo Fisher Scientific) and captured by BioMax film (Kodak, Rochester, NY). For detection of Dll4 Ag and HA-tag on the same immunoblot, we first probed for the presence of Dll4 and stripped the membrane off of all Abs by immersing it in Stripping Buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 0.1 M 2-ME) at 55°C for 1 h. The membrane was then washed extensively in PBS, followed by the standard procedures for Western blot analysis mentioned above for detection of HA tag.

Immuno-fluorescent microscopy

OP9-DL1\(^{22}\) and OP9-DL4\(^{24}\) cells were seeded onto cover slips overnight. Standard procedures were used for staining. Briefly, the cells were fixed with paraformaldehyde EM grade (Electron Microscopy Sciences, Hatfield, PA) diluted to 2% in PBS for 20 min, followed by permeabilization in PBS-T (PBS and 0.05% Triton X-100) for 30 min (Sigma-Aldrich) plus 5% FBS. Primary Abs used were anti-HA and anti-β-tubulin (see above). Appropriate fluorescent-conjugated secondary Abs were applied (Jackson ImmunoResearch Laboratories) to visualize cells under fluorescent light. Images were captured using a Zeiss microscope (Carl Zeiss Canada, Toronto, Ontario, Canada) and analyzed using the AxioVision software (Carl Zeiss). Same time of exposure during the acquisition and same values during processing were applied to each image.

Recombinant Dll-Fc protein generation

DNA encoding the extracellular domains (ECDs) of Dll1 and Dll4 were PCR-amplified using the primers included in the Supplemental Table I and ligated in frame to sequences coding for the Fc region of human IgG3 (Fc\(^y\)) in the plasmid pVL1393 (gift of Dr. Francisco Sanchez-Madrid, Madrid, Spain). The resulting constructs coding for the ECD of Dll1 or Dll4 fused with Fc\(^y\) (Dll1-Fc or Dll4-Fc) were inserted into the multiple cloning site of baculovirus expression vector pAcGP67-A (BD Biosciences) and used to transfect SF9 insect cells. Transfected SF9 cells produced viral particles and secreted the fusion protein that was purified from the supernatant using protein-A purification column (Thermo Fisher Scientific). Dll1-Fc and Dll4-Fc fusion proteins were eluted off the column using low pH solution (20 mM glycine pH 2.8, 0.2 M NaCl) and placed directly in tubes containing 0.1 M Tris pH 8.5 at a 2:1 ratio, respectively. To measure the protein concentration of the purified protein, we compared a standard curve of each solution to standard amounts of BSA through gel electrophoresis and separation of proteins by gel electrophoresis, followed by Coomassie Brilliant Blue (Sigma-Aldrich) staining. We quantified the concentration using GS-800 Densitometer (Bio-Rad).

For cellular binding assay, ex vivo thymocytes derived from E16 thymuses and murine lymphoblast cell lines, EL4 (ATCC) and SL12 were first blocked by 10 min incubation with unconjugated anti-CD16/CD32 mAb (clone 2.4G2). The 1 μg Dll1-Fc or Dll4-Fc was incubated with 60,000 cells in 1 ml HBSS for 30 min while rotating at 4°C. The cells were then washed and secondary fluorochrome-conjugated anti-human Ab (Jackson Immunoresearch Laboratories) was applied for analysis by flow cytometry.

Biotin-labeling of cell-surface proteins

We followed the manufacturer’s instructions for labeling cell-surface proteins (Pinpoint Cell Surface Isolation Kit, Thermo Fisher Scientific). Briefly, OP9-DL1\(^{22}\) and OP9-DL4\(^{22}\) cells were each seeded overnight at near confluency in 6-well plates. The media was removed and the cells were washed twice with cold PBS. The plates were then placed on ice, before adding 650 μl biotin-linking solution (sulfo-NHS-SS-biotin [sulfosuccinimidyl-2-biotinamido ethyl-1,3-dithiopropionate]) to the cells and allowed to rock for 30 min. The reaction was then stopped by removing the biotin-linking solution and was followed by quenching the reaction for 5 min. The cells were then lysed by the addition of cold RIPA buffer and protease inhibitors as described previously. The lysates were removed and their protein concentrations measured using BCA assay (Thermo Fisher Scientific). Equal amounts of protein and volume per sample were added to 25 μl Avidin-conjugated beads. After 1 h of end-to-end mixing with a rotator, the beads were washed and the biotinylated proteins were eluted off the beads using SDS loading buffer (containing a reducing agent 0.1 M DTT) for examination by separation through gel electrophoresis as described under the Western blot analysis section above.

Results

OP9-DL1 and OP9-DL4 both support T cell development

We first directly compared the ability of OP9 cells overexpressing Dll1 or Dll4 (OP9-DL1 or OP9-DL4) (12, 21) to give rise to T cells from fetal liver-derived HPCs. HPC/OP9 cocultures were harvested after 6 and 12 d, and assessed by flow cytometry for the presence of T or B lymphoid and myeloid lineage cells (Fig. 1). At day 6, both OP9-DL1 and OP9-DL4 cocultures contained a similarly high frequency of CD25\(^{+}\) early T lineage cells (∼80%), these included ∼20% CD44\(^{+}\) CD25\(^{+}\) DN2 cells and ∼60% CD44\(^{+}\) CD25\(^{+}\) DN3 cells. In addition, by day 12, DP cells were generated from HPCs at similar rates (∼40%) in both OP9-DL1 and OP9-DL4 cocultures. In keeping with the known functions of Notch signaling in hematopoietic differentiation (24), B cell (CD19\(^{+}\)) and myeloid cell (CD11b\(^{+}\)) development were abrogated in both Dll1 and Dll4 containing cocultures (Fig. 1). In contrast to OP9 cells expressing Dll1 or Dll4, OP9-control (Cntl) (GFP-only) cocultures gave rise to almost exclusively CD19\(^{+}\) cells (>95%) by day 12; whereas, a small frequency (∼4%) of CD11b\(^{+}\) myeloid cells could be observed on day 6. Therefore, when overexpressed by OP9 cells, Dll1 and Dll4 show similar properties in their ability to give rise to T cells and to inhibit B and myeloid lineage development.

Notch ligand expression in the thymus

The above observations are in contrast to the clear genetic evidence showing that Dll4, but not Dll1, is uniquely required to support intrathymic T cell development (4, 6). To address whether this difference is due to the location and/or level of expression of either Dll molecule, we first surveyed the expression of Notch ligand transcripts in thymic stromal cells isolated from day 16 embryos (E16), using semi–qRT-PCR. To evaluate their specificity, we contrasted their level of expression against whole embryo extracts of the same age and unmodified OP9 cells (Fig. 2A). This analysis shows that Dll4 was expressed ∼10-fold higher in thymic stromal cells as compared with the whole embryo; whereas, Dll1 levels were about five times lower in thymic stromal cells than the whole embryo. Transcripts for Jag1 and Jag2 are both expressed in thymic stromal cells, but only Jag1 expression is readily detectable in OP9 cells. Unlike Dll1 and Dll4, overexpression of Jag1 in

FIGURE 1. Dll1 and Dll4 show similar functions when ectopically overexpressed on OP9 cells. Sorted HPCs (CD42\(^{−}\) Sca1\(^{+}\) CD117\(^{−}\)) derived from E15 fetal livers were cocultured with OP9 Cntl, OP9-DL1 or OP9-DL4 cells, harvested after 6 d (A) or 12 d (B), and analyzed by flow cytometry for the surface expression of CD4, CD8, CD25, CD44, CD11b, and CD19, as indicated. The plots include live cells that were gated for CD45\(^{+}\) expression, with percentages shown for each quadrant. For each histogram, RCN is shown. RCN, relative cell number.
OP9 cells does not support T cell development (25); whereas, overexpression of Jag2 in OP9 cells appeared to only enhance the generation of murine NK cells (26). This simple transcript expression analysis supports the interpretation that Dll4 may be more suited to induce intrathymic T cell development simply due to its higher level of expression within thymic stromal cells.

The above analysis was extended to the adult thymus, because unlike the fetal thymus, the spatial distribution of Notch ligand expression within the cortical and medullary regions of the adult thymus can be morphologically defined (27, 28). We tested for the presence of Notch ligands in the adult thymus and made use of laser-capture microdissection to isolate precise subregions within the cortex (SC, subcapsule; OC, outer cortex; IC, inner cortex; PC, pericortex) and medulla (OM; CM, central medulla) and compared each level with that present in the whole medulla (Fig. 2B). Real-time qRT-PCR analysis of each subregion showed that Dll4 transcripts are highly expressed in the SC and OC regions. In contrast, Dll1 is most abundant at the PC region, which is the point of entry of thymus-seeding progenitors (29). It is also possible that the high levels of Dll1 expression in this region is a reflection of the increased presence of endothelial cells and/or dendritic cells (27). In contrast, Jag1 expression is relatively uniform, with Jag1 showing its highest level in the CM; whereas, Jag2 displays two peaks of expression within the IC and OM regions. Based on its potential roles in gd- T cell and NK cell development (26, 30), it is possible that Jag2 expression in these regions may reflect consequential sites for the generation and maturation of these lineages. The observed pattern of Notch ligand expression suggests that Dll4 is broadly and preferentially expressed within cortical regions, where incidentally DN thymocytes expressing high levels of Notch1 are present (31, 32), and thus uniquely situated to induce and support intrathymic T cell development.

Generation of OP9 cells expressing different levels of Dll1 and Dll4

Our above findings show that the expression of Dll1 and Dll4 appear to be dynamically distributed within nonoverlapping regions of the adult thymus, which may allow for specialized functions of each Dll according to its level of expression. With this in mind, we wanted to test whether different levels of Dll1 or Dll4 expression on stromal cells could reveal distinct properties for these otherwise similarly acting Notch ligands. To this end, we engineered Dll1 and Dll4 constructs that shared an HA Ag epitope tag at their C terminus. OP9 cells were retrovirally transduced with these Dll1-HA and Dll4-HA constructs, which also encoded for GPF as part of a bicistronic transcript. Three sets of OP9 cells were isolated based on their low (lo), intermediate (med), and high (hi) levels of GFP expression (OP9-DL1lo/med/hi and OP9-DL4 lo/med/hi), as detected by flow cytometry, and were shown to have a lower GFP level of expression than the original (Ori) OP9-DL1 or OP9-DL4 cells (Fig. 3A). In addition, staining of these cells with Abs specific to Dll1 and Dll4 constructs that shared an HA Ag epitope tag at their C terminus. OP9 cells were retrovirally transduced with these Dll1-HA and Dll4-HA constructs, which also encoded for GPF as part of a bicistronic transcript. Three sets of OP9 cells were isolated based on their low (lo), intermediate (med), and high (hi) levels of GFP expression (OP9-DL1lo/med/hi and OP9-DL4 lo/med/hi), as detected by flow cytometry, and were shown to have a lower GFP level of expression than the original (Ori) OP9-DL1 or OP9-DL4 cells (Fig. 3A). In addition, staining of these cells with Abs specific to Dll1 and Dll4 showed graded levels of expression on the surface OP9-DL1 and -DL4 cells, respectively (Fig. 3A). Although the graded expression of Dll4 can be readily detected on the cell surface, the relatively weak anti-Dll1 Ab provides a less clear separation among the different levels of expression (Fig. 3B).

To further demonstrate and to directly compare the levels of Dll1 and Dll4 expressed by the different OP9 cell subsets, total cell
lysates were analyzed for the relative amount of HA-tagged Dll proteins present within each stromal cell line. The use of a shared HA-tag and equivalent loading of each sample (antitubulin immunoblot, Fig. 3C) allowed us to directly compare the levels of Dll protein expressed within each of the different OP9 cell lines. We observed a clear and direct correlation between the level of HA-tagged Dll proteins and the observed level of GFP expression (Fig. 3A, 3C), such that the intensity of the HA signals detected on the immunoblots increased in a similar stepwise manner as that seen for GFP expression. Although the levels of GFP expression within each graded subset were equivalent (Fig. 3A), we unexpectedly found that the detected levels of HA-tagged Dll were higher within each of the OP9-DL1 cell subsets than in their corresponding OP9-DL4 cells (Fig. 3C). This discrepancy between the levels of GFP and Dll1 versus Dll4 expression may be due posttranslational modifications that differentially affect the half-life of each protein. Of note, the Dll4-HA protein levels in OP9-DL4 med cells were nearly equivalent to the Dll1-HA protein levels present in OP9-DL1 lo cells. Of note, the Dll4-HA protein levels in OP9-DL4 med cells were near equivalent to the Dll1-HA protein levels present in OP9-DL1 lo cells. The fact that both Dll1-HA and Dll4-HA were predominantly localized to the perinuclear area, although more Dll1-HA appeared to be localized at the plasma membrane as compared with Dll4-HA (Supplemental Fig. 1A). We further examined the cell surface localization of Dll1-HA and Dll4-HA in live OP9 cells by biotin-labeling all cell surface proteins, followed by avidin-head-mediated precipitation of cell lysates that are then immunoblotted with anti-HA Abs (Supplemental Fig. 1B). Our results confirmed the presence of higher amounts of Dll1-HA at the cell surface on all of the OP9-DL1 cell subsets (lo-med-hi) as compared with the corresponding OP9-DL4 cells. Strikingly, we noted that the steady-state surface levels of HA-tagged Dll on OP9-DL4hi cells appear to be similar to those detected on OP9-DL1lo cells (Supplemental Fig. 1B). This suggests that Dll4 may be endocytosed at a higher rate than Dll1, which may contribute to its signaling ability.

It was recently shown that Dll4 is the physiologically relevant Notch ligand expressed in the thymus (4, 6). We therefore sought to evaluate which of the new OP9-DL4 cell lines (lo-med-hi) expressed Dll4 at levels similar to those present in TECs. To this end, EpCAM+ TECs were purified by magnetic-assisted cell sorting from E16 thymuses, and their levels of Dll4 expression were compared with OP9-DL4lo/medhi cells (Fig. 3C). This analysis revealed that the OP9-DL4med cells appear to express similar levels of Dll4 as those present in E16 TEC lysates. This was further supported by flow cytometric analysis of TECs (Fig. 3D), in which the levels of Dll4 staining observed on these cells were akin to those seen on OP9-DL4med cells (Fig. 3B). However, one caveat is that we could not directly compare the levels of anti-Dll4 staining on OP9-DL4med cells to TECs due to their differences in autofluorescence. Nevertheless, the mean fluorescence intensity ratio of anti-Dll4:IgG Cntl of TECs was 5.6, similar to the mean fluorescence intensity ratio of 5.9 for OP9-DL4med cells. In addition, although we could detect the presence of Dll1 on OP9-DL1 cells, albeit weakly with the anti-Dll1 mAb used, Dll1 expression could not be detected on TECs, as its fluorescence profile completely overlapped with the IgG Cntl (Fig. 3D).

**Direct comparison of Dll levels for inducing T cell development**

Having established the relative amounts of Dll expression in OP9-DL1lo/medhi and OP9-DL4lo/medhi cells to each other, and to Dll4 expression in TECs, we then compared their efficiency of initiating and supporting T cell development. To this end, we cocultured E15 FL-derived HPCs with each of the OP9-DL1lo/medhi cell lines and harvested the hematopoietic cells on days 6, 9, and 12 for analysis by flow cytometry. The results shown in Fig. 4A illustrate that OP9 cells expressing low levels of either Dll (Dll1lo or Dll4lo) failed to generate T cells, similar to what is seen with the parental OP9 Cntl cells. However, OP9 cells expressing medium levels of Dll were able to induce T lineage differentiation, but with OP9-DL4med cells supporting a more robust and sustained generation of T lineage cells than the OP9-DL4med cells (Fig. 4A, 4B). There were substantially fewer DN2 and DN3 cells in the OP9-DL1med compared with the OP9-DL4med cocultures at day 6, and this difference was further accentuated by day 9 when >50% of the cells were in DN2-
DN3 stage in OP9-DL4<sub>med</sub> cocultures compared with only ~5% in OP9-DL1<sub>med</sub> (Fig. 4A). Analysis at day 12 showed that OP9-DL4<sub>med</sub> cocultures gave rise to a clear population of DP cells as compared with that of OP9-DL1<sub>med</sub> (Fig. 4B, 4C), demonstrating that the medium level of Dll expression in OP9 cells is at the threshold for effective T cell induction by Dll4 but just below the required level by Dll1. Of note, this difference in the induction of T cell differentiation was evident despite the fact that lower levels of Dll4-HA than Dll1-HA were observed by Western blot analysis (Fig. 3C).

The differences in Dll4 versus Dll1 observed at the medium level of expression were not as apparent when cocultures of higher levels of Dll were examined. HPCs cultured on OP9-DL1<sub>hi</sub> and OP9-DL4<sub>hi</sub> were able to generate T lineage cells with similar efficiencies (Fig. 4A). However, there was an increased appearance of the DP cells seen at days 9 and 12 in the OP9-DL4<sub>hi</sub> cells than in the OP9-DL1<sub>hi</sub> cocultures (Fig. 4B, 4C). Collectively, these results suggest that, when expressed at limiting levels, Dll4 retains a greater ability for generating T cells than Dll1.

To determine the underlying reason for the observed differences between Dll1 and Dll4, we investigated their ability to bind to Notch receptors expressed on ex vivo thymocytes and lymphoma cell lines, EL4 and SL12. To this end, we generated Dll1-Fc and Dll4-Fc recombinant fusion proteins that consisted of the extracellular regions of Dll1 or Dll4 fused to the human Fcγ domain, respectively. Flow cytometry analysis showed greater binding by Dll4-Fc to DN thymocytes than the Dll1-Fc protein (Supplemental Fig. 2), consistent with previous reports (20, 33). In addition, Dll4-Fc also showed increased binding to EL4 and SL12 cell lines, compared with Dll1-Fc (Supplemental Fig. 2). However, neither Dll1-Fc nor Dll4-Fc bound to DP thymocytes, which downregulate Notch1 expression (31, 32), indicating that the binding of both Dll1-Fc and Dll4-Fc to DN thymocytes is specific. Conversely, a recent paper using a novel anti-Notch1 mAb showed that low levels of Notch1 could be detected on DP cells (32), indicating that the Dll-Fc recombinant proteins may not be as sensitive. Nevertheless, to evaluate the significance of their binding capacities, we addressed whether Dll1-Fc and Dll4-Fc were functional. Recombinant Dll-Fc proteins, cross-linked with an anti-human Ab, were added to OP9-Cntl/HPC cocultures, and flow cytometric analysis showed that both Dll1-Fc and Dll4-Fc were capable of inhibiting B lineage development and promoting early T lineage generation (data not shown) (33). Altogether, these results demonstrated that recombinantly generated Dll1 and Dll4 fusion
proteins were functional and further suggested that Dll4 interacts more effectively with Notch receptors present on developing T cells than Dll1.

Although Dll4 appears to have a greater effect than Dll1 on the induction of T lineage differentiation from HPCs, we wanted to determine whether this ability was also reflected on the absolute number of cells obtained in vitro. To this end, we determined the absolute number of hematopoietic cells from different cocultures (Fig. 4D). Interestingly, OP9 cell lines expressing Dll1hi or Dll4med/hi, which supported a greater proportion of T lineage cells, also generated the least absolute number of cells. In contrast, the OP9 cell lines expressing Dll1lo/med or Dll4lo, which supported a greater proportion of non-T lineage cells, generated higher absolute number of cells. This difference in cell number likely reflects the ability of graded levels of Dll-mediated signals to support different cell-lineage outcomes, each with apparently varying capacities of cellular expansion.

B cell and myeloid development on OP9-DL cocultures

As pointed out above, the generation of non-T lineage cells is inversely affected by the levels of Dll expression on the OP9 cells. To further explore this, we assessed the ability of graded levels of Dll1 and Dll4 on OP9 cells to inhibit B cell development from HPCs. Flow cytometric analysis at day 6 showed that CD19+ B lineage cells comprise 50% of the hematopoietic cells present in OP9-DL1lo and OP9-DL4lo coculture populations, similar to those observed with parental OP9 Cntl cells, demonstrating that low levels of Dll1 and Dll4 are unable to inhibit B cell development (Fig. 5A, 5B). By day 9, the population of B cells increases to >80% in these cocultures. In contrast, high levels of either Dll1 or Dll4 effectively inhibited the generation of CD19+ B lineage cells, similar to that observed in Fig. 1 and as previously reported (12). However, when OP9 cells expressed Dll at medium levels, we noted a large difference in the generation of B cells, with Dll4med showing a 10-fold increased effectiveness over Dll1med in reducing the number of B cells obtained at days 6 and 9 of coculture (Figs. 4D, 5B). Taken together, these results demonstrate that OP9-DL4med cells, which express Dll4 at levels comparable to those on TECs, support effective T cell differentiation while still inhibiting the B lineage outcome.

In addition, we examined whether the expression of graded levels of Dll1 and Dll4 on OP9 cells affected the development of myeloid-lineage cells from HPCs. Flow cytometric analysis at day 6 showed that CD11b+ myeloid-lineage cells comprised ~15% of the hematopoietic cells present in OP9-DL1lo and OP9-DL4lo coculture populations, similar to those observed with parental OP9 Cntl cells, demonstrating that low levels of Dll1 and Dll4 are permissive for myeloid cell development (Fig. 5C, 5D). By day 9, the proportion of CD11b+ cells in these cocultures had slightly decreased. In contrast, high levels of either Dll1 or Dll4 effectively inhibited the generation of CD11b+ myeloid-lineage cells, similar to that observed in Fig. 1 and as previously reported (15). However, when OP9 cells expressed Dll at medium levels, we noted a clear increase in the generation of myeloid cells as

**FIGURE 5.** Different levels of Dll1 and Dll4 expressed on OP9 cells support the generation of diverse cell fates from HPCs. A, Flow cytometric analysis for the expression of CD19 at days 6 and 9 of coculture, with different OP9 cell lines, as indicated. B, The average percentage of CD19+ cells present at days 6 and 9 of coculture with the indicated OP9 cell lines is shown from three independent experiments (error bars represent SEM; lo [l], med [m], hi [h], OP9-DL1 [DL1], OP9-DL4 [DL4]). C, Flow cytometric analysis for the expression of CD11b at days 6 and 9 of coculture, with different OP9 cell lines, as indicated (top two rows). Bottom row shows cells from OP9, OP9-DL1med, and OP9-DL4med cocultures that were electronically gated as CD11b+ or CD11b− (dashed lines within histogram) and further analyzed for the expression of CD45R (B220) and CD11c as indicated by dashed arrows. D, The average percentage of CD11b+ cells present at days 6 and 9 of cocultures with the indicated OP9 cell lines is shown from three independent experiments (error bars represent SEM; lo [l], med [m], hi [h], OP9-DL1 [DL1], OP9-DL4 [DL4]). E, The average total cellularity of pDCs (CD11b+ CD11c+ CD45R+) measured at day 9 of cocultures with the indicated OP9 cell lines is shown from three independent experiments (error bars represent SEM).
compared with either low or high levels (Fig. 5D), and surprisingly both Dll4med and Dll1med were similarly permissive in allowing the differentiation of myeloid cells. Notably, the absolute number of myeloid cells generated on medium levels of either Dll1 or Dll4 was significantly higher than those from OP9 cells expressing low levels. Interestingly, further analysis revealed the appearance of CD11b+CD11c+CD45R+ myeloid cells, representing a plasmacytoid dendritic cell (pDC) population, whose presence is enhanced in cocultures containing medium or low levels of Dll, but not found when Dll1 and Dll4 are absent or expressed at high levels (Fig. 5C, lower insert, 5E). These findings illustrate that Dll molecules appear to acquire the capacity to give rise to specific cell lineages depending on their particular level of expression, which may reflect differential thresholds for the induction of Notch target genes.

Levels of Dll expression and lineage-related gene expression

To determine the intensity of Notch signaling induced in HPCs by OP9 cells expressing different amounts of Dll1 and Dll4, we measured the transcript levels of Notch-regulated genes, Deltex1 (Dtx1), Nrarp, and Gata3 (34–36), using qRT-PCR (Fig. 6). HPCs cocultured on the various Dll-expressing OP9 cells were harvested after 48 h mRNA was extracted and reverse transcribed to cDNA for qRT-PCR analysis. The results shown in Fig. 6 confirmed that high levels of Dll4 expression in OP9 cells leads to a clear induction of Dtx1 and Nrarp expression (90- and 21-fold higher than OP9-Cntl, respectively); whereas, Gata3 induction was less apparent. Surprisingly, HPCs cocultured on OP9-DL1 hi cells showed statistically significantly lower levels of Dtx1 and Nrarp gene expression than on OP9-DL4 hi cells, despite their similar ability in inducing early T cell development (Fig. 4A). In contrast to OP9-DL1 hi cells, analysis of the OP9 cells expressing medium and low levels of either Dll1 or Dll4 showed dramatically reduced induction of Dtx1 and Nrarp gene expression. Nevertheless, OP9-DL4med cells showed higher levels of induced Nrarp expression than OP9-DL1med cells, and approached the levels induced by OP9-DL1hi cells. This is reflected in the superior ability of OP9-DL4med cells to support T cell development than OP9-DL1med cells, although less effective than OP9-DL1hi cells (Fig. 4A). Lastly, both OP9-DL1lo and OP9-DL4lo cells showed similarly low but measurable levels of Dtx1 and Nrarp gene induction. These findings support the notion that Dll4 is more effective in activating Notch receptors in HPCs, which correlates to its increased capacity to induce T cell differentiation at limiting levels.

We further examined the effect of graded levels of Dll-mediated signaling on the transcription of the B cell-specific gene, Pax5 (37), in the cocultures. As expected, Pax5 transcripts were not detected from OP9-DL1 hi and OP9-DL1 hi cocultures (Fig. 6) (38), consistent with the absence of B cell development in these cultures. However, Pax5 transcript levels in cells cultured on OP9-DL1 lo cells approached those from OP9-Cntl cocultures; whereas, on OP9-DL4 hi cells the levels amounted to only ∼40% of Cntl (Fig. 6), even though both cocultures can give rise to >80% B cells (Fig. 5A). In addition, a further drop in Pax5 transcripts levels, down to 20% of Cntl, was seen in OP9-DL1 med cocultures, which like Dll lo cocultures, are also permissive to generate B cells. In contrast, OP9-DL4 med cocultures showed a stronger, near complete, reduction in Pax5 transcripts, keeping with the much greater inhibition in B lymphopoiesis than in OP9-DL1 med cultures (Fig. 5A, 5B).

Although Notch-regulated genes, Dtx1 and Nrarp, and Pax5 transcript levels correlated well with T versus B lymphopoiesis, the levels of the myeloid lineage transcription factor, Cebpα (39), did not relate directly to the number of myeloid cells obtained. For instance, although higher levels of Cebpα transcripts were...
detected from HPCs cultured on OP9-DL<sup>lo</sup> cells, as compared with OP9-DL<sup>med</sup> cells (Fig. 6), the DI<sup>lo</sup> cultures did not generate more myeloid CD11b<sup>+</sup> cells than DI<sup>med</sup> cultures (Figs. 4D, 5D). Nevertheless, OP9-DL<sup>lo</sup> cultures contained greater numbers of CD11b<sup>+</sup> CD11c<sup>+</sup> CD45<sup>R<sup>2</sup></sup> pDCs (Fig. 5C, 5E), and showed a corresponding increase in the levels of Spi<sub>b</sub> transcripts (Fig. 6), which plays an important role in pDC differentiation (40). Medium levels of DI expression also supported the generation of pDCs. These results suggest that low to medium levels of DI expression appear to be preferentially permissive for specific myeloid outcomes.

Discussion

DI<sub>4</sub> is more efficient than DI<sub>1</sub> in T lymphopoiesis induction

The role of DI Notch ligands in inducing T cell development in vitro is now well established, as previous work showed that ectopic overexpression of DI1 or DI4 in OP9 cells (5, 12, 20), or thymic stromal cells (16), is sufficient to support T lineage commitment and differentiation from HPCs. In keeping with these findings, when we used OP9 cells expressing very high levels of either DI1 or DI4, similar to that expressed in the Ori OP9-DL1 cells (12), we could not distinguish between their ability to give rise to T lineage cells or inhibit B and myeloid cell differentiation (Fig. 1). This suggested that these DIls could be functionally equivalent and/or that their expression levels in OP9 cells reached saturation levels for activating Notch receptors on HPCs. However, in light of the recent findings showing that DI4 is uniquely required for T lymphopoiesis in vivo (4–6), we hypothesized that if equally expressed at near physiological levels in vitro, we would then discern a functional difference for DI1 and DI4 in activating the Notch-dependent program of T lineage differentiation from HPCs.

To this end, we generated a new set of OP9 cell lines expressing discrete and comparable levels of HA-tagged DI1 and DI4 proteins, which were lower than that expressed by the Ori OP9-DL1 or OP9-DL4 cells (Fig. 3). These cells allowed us to demonstrate that despite lower cell surface protein levels of DI4-HA present on OP9-DL4<sup>hi</sup> and OP9-DL4<sup>med</sup> cells compared with the levels of DI1-HA on OP9-DL1<sup>hi</sup> and OP9-DL1<sup>med</sup> respectively, the DI4-expressing cells were more effective in supporting T cell generation (Fig. 4). The OP9-DL4<sup>hi</sup> and OP9-DL4<sup>med</sup> cells also showed higher induction of Notch target genes as compared with their OP9-DL1 counterparts (Fig. 6). These results demonstrate that when directly compared with DI1, DI4 possesses a greater capacity to activate the Notch pathway in HPCs.

Notch activation by DI4 is dependent on two discernible events. First, the ECD of DI4, specifically the Delta-Serrate-Lag2 region, engages Notch receptors. Second, this interaction allows DI4, while undergoing active endocytosis, to pull on the Notch-ECD exposing a proteolytic site that is cleaved for its activation (41). We found that a recombinant DI4-Fc protein showed greater avidity than DI1-Fc for binding DN thymocytes (Supplemental Fig. 2), and as reported by others (20, 33). This is despite the evidence that DI4 interacts with Notch1 but not Notch2, whereas, DI1 can interact with both Notch receptors (20). In addition, the lower levels of DI4 than DI1 detected on the cell surface of OP9 cells (Supplemental Fig. 1) suggests that DI4 may have a higher rate of endocytosis, leading to more effective pulling on Notch for its activation. Thus, the high-avidity binding of DI4 to DN thymocytes and its potential to more effectively trans-endocytose Notch-ECD, coupled with results showing strong Notch activation at the molecular level (Fig. 6), provide a compelling mechanism for the greater capacity of DI4 to induce T cell development.

Expression of DI4 on OP9 cells, at levels found on TECs, supports the development of T and myeloid cell lineages

OP9-DL4<sup>med</sup> cells and fetal TECs were found to express comparable amounts of DI4, allowing us to more closely replicate the levels of Notch signaling that are likely to be induced in progenitors within the thymic microenvironment. HPCs cultured with OP9-DL4<sup>med</sup> cells showed that, like the thymus, these cultures yielded mostly T lineage cells, inhibited B lineage but allowed for myeloid cells to be generated. Recent reports have shown that early thymic progenitors have the ability to give rise to myeloid lineage cells, but show limited B lineage potential (42–44). Our findings using the OP9-DL4<sup>med</sup> cells revealed that at this level of DI4-mediated signals the outcomes obtained in vitro are in line with the demonstrated lineage potential displayed by early progenitors within the thymus. In addition, this permissive level of DI4-mediated Notch signals may explain the thymus' ability to give rise not only to T cells, but pDCs as well (45, 46).

Our results also predict that if DI4 was to be expressed at higher levels in the thymus, or if progenitors were to become more sensitive to Notch signals, this may lead to a complete inhibition of pDC development or loss of myeloid potential by early progenitors. This prediction relies on the notion that the basis for commitment to a particular lineage can be attributed to the balance of transcription factors that are required to specify or inhibit lineage outcomes (47). Thus, the near exclusive generation of T lineage cells from OP9-DL4<sup>lo</sup> cocultures is likely due to the high levels of activated intracellular Notch leading to the repression of Pax5 and Cebpα expression (Fig. 6). In contrast, the generation of B cells in OP9-DL4<sup>lo</sup> cocultures can be explained by the high levels of Pax5 expression, acting to repress Notch1 expression (48) and inhibit C/EBPα function (49). However, the increased generation of myeloid cells in OP9-DL4<sup>med</sup> suggests that sufficient Notch signaling activation results in Pax5 repression, which together with the available, albeit lower, levels of Cebpα provides a permissive transcriptional program to allow for myelopoiesis.

Previous work has demonstrated the effects of differential Notch activation and the lineage outcomes of cocultured HPCs using increasing doses of γ-secretase inhibitor (50–52). However, those studies examined a single Notch ligand (DI1) and did not compare the relative capabilities of DI1 and DI4 to promote T lineage differentiation at given amounts of the inhibitor. Nevertheless, this approach, together with our findings, show that at discrete and varying levels of Notch signaling, either by limiting activation of the receptor or by reducing the ligand availability, there is an apparent hierarchical set of lineage outcomes.

Taken together, the current study provides clear evidence showing that at limiting or physiological levels of expression, DI4 is better suited for inducing T lymphopoiesis in vitro. Furthermore, our findings suggest that OP9-DL4<sup>med</sup> cells may offer a more accurate reflection of the Notch signals that are induced within the thymus, which initiate a T cell developmental program, although still permitting limited myelopoiesis, from a diverse pool of incoming progenitor cells.

Acknowledgments

We thank Gisele Knowles and Arian Khandani for expert assistance with flow cytometry, cell sorting, and immunofluorescent microscopy. We thank Dr. Minghong Yan (Genentech, CA) for providing the anti-Dll1 mAb and Dr. Hugh Robson MacDonald (Ludwig Institute for Cancer Research, Zurich, Switzerland) for providing the anti-Dll1 mAb.

Disclosures

The authors have no financial conflicts of interest.
References


Corrections


In Materials and Methods, under the heading OP9-DL1(lo/med/hi) and OP9-DL4(lo/med/hi) cells, in both the penultimate sentence and the last sentence, “GP” and E86” and “GP and E86” should be “GP+E86”. In addition, under the heading Western blot analysis, in the first sentence, “0.5% doxycycline” should be “0.5% Na deoxycholate”.

In Results, under the heading Generation of OP9 cells expressing different levels of Dll1 and Dll4, in the fourth sentence of the first paragraph, “GPF” should be “GFP”. In the fifth sentence of the third paragraph, “immunobloted” should be “immunoblotted”.

In Figs. 3C, 5A, and 5C, the labels for the immunoblots and flow cytometry plots were mistakenly duplicated as OP9-DL1. The labels on the left are correct as “OP9-DL1”, but the labels on the right should be “OP9-DL4”. The results and the conclusions in the article remain unchanged. The corrected figures are shown below. The published figure legends are correct but are shown below for reference.

The online version of this article has been corrected and now differs from the print version as originally published.

FIGURE 3. Generation of OP9 cells expressing discrete and comparable levels of Dll1 and Dll4. A, OP9 cells were retrovirally transduced and then sorted based on different levels of GFP fluorescence. Flow cytometric analysis for GFP expression is shown for OP9-DL1 (lo/med/hi) or OP9-DL4 (lo/med/hi), parental OP9 cells (OP9), and the originally described OP9-DL1 (Ori) and OP9-DL4 (Ori) cells. B, Flow cytometric analysis is shown for cell surface expression of Dll1 and Dll4 on OP9-DL1 (lo/med/hi), OP9-DL4 (lo/med/hi), and parental OP9 cells. C, Immunoblot analysis of OP9-DL1 (lo/med/hi), OP9-DL4 (lo/med/hi), parental OP9 cells, E16 total thymus, and purified TEC samples probed for the expression Dll4 and β-tubulin. Blots were stripped and reprobed for HA-tagged Dll1 and Dll4 expression. Anti–β-tubulin Ab was used to detect equal loading per lane for the immunoblot. The blot shown is representative of four independent experiments. D, Flow cytometric analysis is shown for E16 TECs and OP9-DL4 (med) cells for Dll4 and Dll1 (TEC only) expression, with cells not stained with primary Ab shown as Cntl.
FIGURE 5. Different levels of Dll1 and Dll4 expressed on OP9 cells support the generation of diverse cell fates from HPCs. A, Flow cytometric analysis for the expression of CD19 at days 6 and 9 of coculture, with different OP9 cell lines, as indicated. B, The average percentage of CD19+ cells present at days 6 and 9 of cocultures with the indicated OP9 cell lines is shown from three independent experiments (error bars represent SEM; lo [l], med [m], hi [h], OP9-DL1 [DL1], OP9-DL4 [DL4]). C, Flow cytometric analysis for the expression of CD11b at days 6 and 9 of coculture, with different OP9 cell lines, as indicated (top two rows). Bottom row shows cells from OP9, OP9-DL1med, and OP9-DL4med cocultures that were electronically gated as CD11b+ or CD11b+ (dashed lines within histogram) and further analyzed for the expression of CD45R (B220) and CD11c as indicated by dashed arrows. D, The average percentage of CD11b+ cells present at days 6 and 9 of cocultures with the indicated OP9 cell lines is shown from three independent experiments (error bars represent SEM; lo [l], med [m], hi [h], OP9-DL1 [DL1], OP9-DL4 [DL4]). E, The average total cellularity of pDCs (CD11b+ CD11c+ CD45R+) measured at day 9 of cocultures with the indicated OP9 cell lines is shown from three independent experiments (error bars represent SEM).
Supplemental Figure 1. Localization of Dll1-HA and Dll4-HA proteins in OP9 cells. A) OP9-DL1^hi and OP9-DL4^hi cells were permeabilized and stained for HA (red) and tubulin (green) expression, as indicated. The images chosen are representative of images of cells that show similar levels of anti-HA perinuclear staining. B) To compare the presence of Dll1 and Dll4 on the surface of OP9-DL1^{lo/med/hi} and OP9-DL4^{lo/med/hi} cells, non-permeabilized cells were biotin labeled (as illustrated), followed by cell lysis and precipitation of biotin-labeled proteins using avidin-conjugated beads. Biotin-labeled proteins were released from the beads by the reducing agent DTT present in SDS loading buffer. Proteins were separated by gel electrophoresis and western blot analysis for Dll1-HA and Dll4-HA proteins present in each sample is shown. Data is representative of 3 independent experiments.
**Supplemental Figure 2.** Higher avidity of Dll4 than Dll1 for Notch expressed on DN thymocytes. Flow cytometric analysis of EL4, SL12 cell lines, and E16 ex vivo thymocytes stained using recombinant Dll1-Fc and Dll4-Fc proteins, detected with APC-conjugated anti-human IgG Ab. Thymocytes were also stained with anti-CD4 and anti-CD8 to allow for the DN and DP gating. Representative flow cytometry histograms show the binding of control anti-human IgG only (Cntl, red line), Dll1-Fc (blue line) and Dll4-Fc (green line) to the different cells as indicated.
**Supplemental Table I.** List of oligonucleotide primers used for qRT-PCR analysis, and for the generation of the Dll-HA tagged constructs.

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Tabular representation of primers used in the study.