HEBAAlt enhances the T-cell potential of fetal myeloid-biased precursors

Marsela Braunstein¹,², Paula Rajkumar¹, Carol L. Claus¹,³, Giovanna Vaccarelli¹,²,⁴, Amanda J. Moore¹,², Duncheng Wang¹,²,⁵ and Michele K. Anderson¹,²

¹Division of Molecular and Cellular Biology, Sunnybrook Research Institute, Toronto, Ontario M4N 3M5, Canada
²Department of Immunology, University of Toronto, Toronto, Ontario M5S 1A8, Canada
³Present address: Sanofi-Pasteur, Toronto, Ontario M2R 3T4, Canada
⁴Present address: Department of Genetics and Microbiology, University of Bari, Bari 70126, Italy
⁵Present address: Canadian Blood Services, UBC Centre for Blood Research, Vancouver, British Columbia V6T 1Z3, Canada

Correspondence to: M. K. Anderson, Sunnybrook Research Institute, 2075 Bayview Avenue, Room A340, Toronto, Ontario M4N 3M5, Canada; E-mail: manderso@sri.utoronto.ca

Received 23 June 2010, accepted 6 October 2010

Abstract

Hematopoiesis is controlled by the interplay between transcription factors and environmental signals. One of the primary determinants of the T-lineage choice is Delta-like (DL)-Notch signaling, which promotes T-cell development and inhibits B-cell development. We have found that the transcription factor HEBAlt is up-regulated in early hematopoietic precursors in response to DL-Notch signaling and that it can promote early T-cell development. Here, we identified a population of lineage-negative Sca-1⁻² c-kit¹ (LK) cells in the mouse fetal liver that rapidly gave rise to myeloid cells and B cells but exhibited very little T-cell potential. However, forced expression of HEBAlt in these precursors restored their ability to develop into T cells. We also showed that Ikaros and Notch1 are up-regulated in response to HEBAlt over-expression and that activated Notch1 enhances the ability of LK cells to enter the T-cell lineage. Furthermore, the myeloid transcription factor C/EBPα is down-regulated in response to HEBAlt. We therefore propose that HEBAlt plays a role in the network that enforces the T-lineage fate and limits myeloid fate during hematopoiesis.

Keywords: E-proteins, myeloid, Notch1, T-cell development, transcription factors

Introduction

T cells are generated from hematopoietic stem cells (HSCs) through developmental intermediates that have increasingly restricted lineage potential, but the gene regulatory networks that guide this process are not well understood. Development of these precursors into T cells requires Delta-like (DL)-Notch1 signaling, which initiates the T-lineage gene program and inhibits factors that direct alternative lineage potential (1, 2). DL-Notch1 signaling is also required for survival and continued T-cell differentiation throughout early development but is not strictly needed once cells become T-lineage committed (3). The early thymic precursors (ETPs) have very little B-cell potential but retain the ability to become dendritic cells, NK cells and myeloid cells, in addition to T cells. Initiation of the T-cell gene program, referred to as specification, is characterized by up-regulation of CD25 and Thy1, resulting in DN2 (CD44⁺CD25⁻Thy1⁺) and DN3 (CD44⁺CD25⁻Thy1⁺) cells (4, 5). At the DN3 stage, a signal must be received through either the pre-TCR or the γδ-TCR for survival and progression to the next stages of αβ or γδ T-cell development, respectively (6).

A variety of hematopoietic progenitors give rise to T cells, including common myeloid lymphoid progenitors and LMPPs (lymphoid-primed multipotent progenitors) (7–10). LMPPs, which have lymphoid and myeloid potential but lack erythroid potential, require both E2A and Ikaros to turn on lymphoid genes and limit the expression of myeloid genes (11). Resolution of lineage potential, however, requires additional waves of transcription factors to build the appropriate positive and negative feedback loops necessary for lineage commitment (12–15). E2A belongs to the E-protein family of transcription factors, which also includes HEB (HEBAAlt and HEBCan) and E2-2 (E2-2Alt and E2-2Can) (16). When DL-Notch signaling is present, E2A is directed to T-cell specific targets, including Notch1 itself and Notch1 target genes such as Hes-1 (17, 18). In the absence of DL, however, E2A turns on the essential B-cell regulators EBF, Pax5 and IL7R, which potentiate E2A activity on B-cell targets (19–21). The mechanisms by which E-protein activities are interpreted in different lineages, however, are not well understood.
HEBAlt restores T-cell potential

E-proteins function as homodimers or as heterodimers with many other types of basic helix-loop-helix factors. E2A and HEB are both expressed during T-cell and B-cell development, and each appears to have distinct as well as overlapping roles in each lineage (16). However, the roles of the HEB factors are much less clear than those of E2A. We previously identified the E-protein HEBAlt, which differs from HEBCan by the presence of a small alternative (Alt) domain that replaces the longer N-terminus found in HEBCan. We and others have shown that HEBAlt is specifically induced in response to DL-Notch signaling and down-regulated at β-selection and that it promotes early T-cell development (22, 23). HEBCan is also expressed in early T-cell precursors, but instead of enhancing T-cell specification, HEBCan inhibits it. These results suggest that one role for HEBAlt could be to direct E2A toward T-cell specific targets such as Notch1. Another potential role could be to antagonize regulators of other lineages that would cause down-regulation of Notch1. To assess these possibilities, we tested the ability of HEBAlt to influence the development of myeloid precursors into T-lineage cells. Remarkably, introduction of HEBAlt into these cells inhibited myeloid development and restored the ability of LK cells to become T-lineage cells. Moreover, Ikaros and Notch1 were both up-regulated in response to HEBAlt, whereas C/EBPα was down-regulated. Since HEBAlt is expressed in pro-T cells, which retain latent myeloid potential, our results suggest that one role of HEBAlt may be to suppress myeloid development in developing T-cell precursors.

Methods

Mice, cells and cell lines

WT C57Bl/6 mice were maintained in the comparative facility at Sunnybrook Research Institute (SRI) and set up for timed mating to generate E14.5 embryos. For some experiments, CD1 timed-mated females were ordered from Charles River (St. Constant, Quebec, Canada). All animal protocols were approved by the animal care committee at SRI. OP9-GFP and OP9-DL1 stromal cells (obtained from J. C. Zúñiga-Pflücker, SRI and University of Toronto, Toronto, Ontario, Canada) were maintained and co-cultured with HSCs in OP9 medium (24).

Isolation of fetal liver hematopoietic precursors

E14.5 fetal livers were disaggregated into single-cell suspensions and incubated with antibodies against F4/80, Gr1, Ter119 and CD19 to obtain lineage-negative (lin−) cells. Lin− cells were enriched using streptavidin-conjugated magnetic beads and MACS separation columns (Miltenyi Biotec, Auburn, CA, USA). For some experiments, CD24+ cells were depleted using anti-CD24 (J11D) and Low-Tox-H Rabbit complement and then isolated using Lympholyte M prior to MACS separation. Lin− cells were cultured overnight in OP9 medium supplemented with 10 ng ml−1 IL-7, Flt3L and SCF (R&D Systems, Burlington, Ontario, Canada). Hematopoietic precursors were sorted using a FACSDiva cell sorter or FACSAria (Becton-Dickinson, Mississauga, Ontario, Canada) for LSK (lin−, Sca-1+CD19−), LK (lin−, Sca-1+CD19−), pre-pro-B (linB220−/CD45+CD19−), early pre-pro-B (CD45+CD19−) or myeloid cells (CD11b+CD11c−). All cells were DAPI or propidium iodide negative. Antibodies were purchased from eBiosciences (San Diego, CA, USA), Becton-Dickinson or the SRI hybridoma facility. Flow cytometry was performed using FACSCalibur or LSIII flow cytometers (Becton-Dickinson). Data were analyzed with FlowJo (Tree Star, Ashland, OR, USA) software.

Retroviral transductions

For HEBAlt, HEBCan and HEBTr transduction experiments, supernatants were prepared as previously described (22). For ICN (intracellular Notch) transduction experiments, Platinum-E packaging cells (Cell Biolabs, San Diego, CA, USA) were transfected with either MIGR1-ICN (25) or MIGR1-control constructs using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Supernatants were collected at 48 h, supplemented with Lipofectamine2000 and used for transduction of LK cells by spin-infection. The cells were cultured overnight in media supplemented with 10 ng ml−1 IL-7, SCF and Flt3L. In some experiments, Lin− cells were co-cultured overnight with stably transfected GP+E packaging cells containing HEBAlt- or HEBCan-encoding retroviral vectors in OP9 medium supplemented with 10 ng ml−1 IL-7, SCF and Flt3L. The GFP+ LSK and/or LK fractions were sorted and cultured in media on OP9-DL1 stromal cells or on OP9-GFP stromal cells.

In vitro cultures

LSK or LK cells were cultured with OP9-DL1 cells, OP9-GFP cells or in media with the following cytokines: (1) for B or T lineage (co-cultures): 5 ng ml−1 IL-7, 5 ng ml−1 Flt3L, 5 ng ml−1 SCF, (2) for B/myeloid lineage (in solution): 10 ng ml−1 IL-7, 10 ng ml−1 Flt3L, 10 ng ml−1 SCF and 20 ng ml−1 granulocyte macrophage colony-stimulating factor (GM-CSF). Cells were plated at approximately 3000–10 000 cells per well, keeping all cell numbers identical within each experiment. Co-cultures were passed onto fresh stroma every 3–4 days. For some experiments, cells were placed in hanging drop fetal thymic organ cultures (FTOCs) for 2 days followed by traditional raft-based FTOC for 7–14 days. No exogenous cytokines were added to the FTOCs.

Real-time PCRs

Total RNA was isolated with Trizol (Invitrogen) and converted into cDNA using Superscript III (Invitrogen). Quantitative real-time reverse transcription–PCRs (Q-PCRs) were performed on cDNA templates using SYBR Green (Bio-Rad, Hercules, CA, USA) software. Data were analyzed with FlowJo (Tree Star, Ashland, OR, USA) software.

Results

The fetal liver LK population contains primarily myeloid-primed and B lineage-primed precursors

One role for HEBAlt in early T-cell development could be to maintain T-lineage identity by suppressing alternative lineage potential. ETPs have very little B-cell potential but do

Downloaded from https://academic.oup.com/intimm/article-abstract/22/12/963/701005 by guest on 20 November 2018
have considerable myeloid potential (26, 27). Therefore, we
decided to test the possibility that HEBAlt could influence
the T/myeloid lineage choice by retrovirally forcing its ex-
pression in myeloid progenitors and assessing the develop-
mental outcomes. Although myeloid cells can develop via
multiple pathways, one common defining feature of all my-
eloid precursors is their expression of c-kit (28–32). Retroviral
transduction requires overnight culture with cytokines (IL-7,
SCF and Flt3L) to allow detection of the green fluorescent
protein (GFP) marker that is co-expressed with HEBAlt in
transduced cells. We therefore first tested the lineage poten-
tial of precursors after overnight culture to determine
whether lineage potential is altered during the culture period.
Lineage-negative (lin-) E14.5 fetal liver cells were obtained
by MACS depletion of cells expressing CD19, Gr1, Ter119
or F4/80 and cultured overnight in 10 ng ml⁻¹ each of IL-7,
SCF and Flt3L. We then sorted LK (lin-, Sca-1⁻ and c-kit⁺)
cells, thought to be comprised of primarily common myeloid
progenitors and LSK (lin-, Sca1⁺ and c-kit⁺) cells, which in-
clude HSCs and LMPPs (10) (Fig. 1A). We compared their
lineage potential by placing them under conditions that pro-
moted B-cell, myeloid and/or T-cell development. In the
presence of GM-CSF, IL-7, SCF and Flt3L, LK cells primarily
adopted a CD11b⁺ myeloid fate (Fig. 1B), whereas LSK cells
became CD19⁺ B cells, consistent with previous observa-
tions (11). In OP9-DL1 co-culture, LSK cells gave rise to
CD25⁺Thy1⁺ T lineage cells, as expected (24), whereas LK
cells were severely restricted from becoming T cells (Fig.
1C). Cell numbers were lower in LK cultures than in LSK cul-
tures (Fig. 1D). Since the LK population contains erythroid
as well as myeloid precursors, this may have been due in
part to the death of erythroid precursors in the absence of
erythropoietic cytokines (31).

LK cells had considerable B-cell potential when GM-CSF
was absent in solution culture and when plated on OP9-GFP
cells (Supplementary Figure S1 is available at Interna-
tional Immunology Online). B220⁺CD11b⁺ cells arose early in cul-
ture, but these populations resolved into B220⁺CD11b⁻ and
B220⁻CD11b⁺ cells by day 7. By day 11 of culture CD11b⁺
cells were not detectable in the LK cultures, which contained
only B220⁺ cells. All B220⁺ cells in day 11 cultures were also
CD24⁺, indicating commitment to the B-cell lineage (data not
shown). LSK cells developed more slowly on OP9-GFP cells
than LK cells, suggesting that at least some LK cells had

![Fig. 1.](https://academic.oup.com/intimm/article-abstract/22/12/963/701005)

Fetal liver LK population contains myeloid-primed and B-lineage-primed precursors. Fetal liver cells were obtained from E14.5 embryos,
depleted of lineage-positive cells (lin = CD19, Gr1, Ter119, F4/80) and cultured overnight in 10 ng ml⁻¹ of SCF, IL-7 and Flt3L. (A) Cultured lin⁻
cells were sorted for the lin⁻Sca-1⁻c-kit⁺ (LSK) and lin⁻Sca-1⁺c-kit⁺ (LK) fractions, and these cells were cultured in conditions promoting (B)
myeloid and B-cell development (10 ng ml⁻¹ SCF, IL7 and Flt3L and 20 ng ml⁻¹ GM-CSF for 6 days) or (C) T-cell development (OP9-DL1 stroma
with 5 ng ml⁻¹ SCF, IL7 and Flt3L for 5 days) prior to analysis by flow cytometry. (D) Cell numbers were counted at the time of flow cytometry for
each set of samples and are represented as mean ± SD. Cell numbers are shown for the conditions listed above, and for LSK versus LK, cells
cultured in 10 ng ml⁻¹ IL-7, SCF and Flt3L, which yield primarily B cells. Cultures of these subsets in OP9-GFP are shown in Supplementary
Figure 1, available at International Immunology Online.
already initiated a B-lineage gene expression program. It should be noted that some T-cell potential was also seen in LK cells that had not been cultured overnight (data not shown), consistent with recent results showing that granulocyte-macrophage precursors have residual T-cell and B-cell potential (11).

**LSK and LK cell populations exhibit different patterns of transcription factor expression**

To understand the gene regulatory environment of LSK and LK cells obtained after overnight culture, we compared their expression of lineage-specific transcription factors (Fig. 2). LSK and LK cells were prepared from E14.5 lin<sup>-</sup> fetal liver cells after overnight culture and subjected to Q-PCR (Fig. 2A). Consistent with a myeloid bias, PU.1 and C/EBPα were expressed at higher levels in LK cells than LSK cells (Fig. 2B). By contrast, levels of Ikaros, EBF and Notch1 were lower in LK cells than LSK cells. Unexpectedly, E2A and HEBCan were higher in LK cells, whereas HEBAlt was higher in LSK cells. The E-protein antagonist Id2 was roughly equivalent in LSK and LK cells. Therefore, the LK cells may exist in a state in which cells are poised to become B cells or myeloid cells, depending on the availability of lineage-specific cytokines. These cells may also comprise a heterogeneous mix of B-lineage and myeloid lineage precursors with different kinetics of development resulting in the early appearance of myeloid cells, followed by a later surge of B-lineage cells (Supplementary Figure S1 is available at *International Immunology* Online). Interestingly, the factor GATA3, which can promote either T-cell or mast cell development, is present at similar levels in both LSK and LK cells. Gfi-1, which promotes B cell or granulocyte fates based on the presence or absence of Ikaros, is also similar in LSK and LK cells. Another lymphoid-biased factor, Runx1, is higher in LK cells. Therefore, the low levels of Ikaros and Notch1 in LK cells may have accounted in part for the low T-cell potential, whereas the high levels of E2A, Gfi-1 and Runx1 in LK cells may have provided latent B-cell potential.

![Fig. 2](https://academic.oup.com/intimm/article-abstract/22/12/963/701005)

**Fig. 2.** Differential expression of regulatory genes in LSK and LK populations. (A) Diagram of cell preparation prior to sorting. (B) Q-PCR analysis of gene expression in LSK and LK populations sorted as shown in (A). The values were calculated using the ΔC<sub>t</sub> method, normalized to β-actin and are presented as the relative mean ± SD of triplicate readings from at least three experiments.
that could be awakened by the addition of IL-7 in the absence of GM-CSF.

**HEB factors inhibit B-cell development from LK cells**

We have previously shown that HEBAlt strongly inhibits B-cell development from LSK cells in a DL-Notch independent manner, whereas it does not impair the survival or differentiation of committed B-cell progenitors (33). To evaluate whether the LK cell population contained committed B-cell precursors that were resistant to HEBAlt, we transduced Lin− cells overnight by co-culturing them on stably transduced GP + E packaging cells expressing control or HEBAlt-encoding retroviral vectors. The LK populations were sorted the following day and co-cultured with OP9-GFP stromal cells supplemented with IL-7, SCF and Flt3L (Fig. 3). After 8 days of co-culture, nearly all of the control-transduced cells developed into CD19+ committed B-cell precursors (Fig. 3A and C). After 8 days of co-culture, nearly all of the control-transduced cells developed into CD19+ committed B-cell precursors (Fig. 3A and C). Moreover, they had expanded ~4-fold (Fig. 3B). These results are consistent with our previous observations in untransduced LK cells on OP9-GFP cells (Supplementary Figure S1 is available at International Immunology Online and data not shown). However, HEBAlt-transduced LK cells failed to up-regulate CD19, and the majority of the cells died. Some of the remaining cells in the HEBAlt-transduced cultures were CD11b+, but most lacked the lineage markers we examined (Thy1, CD25, CD19 and CD11b). Cells transduced with HEBCan also failed to proliferate or develop into B-cell precursors, consistent with our previous finding that HEBCan inhibits B-cell development in the presence of endogenous HEBAlt (33). These results therefore confirmed that HEBAlt can inhibit B-cell development even in precursors that are primed toward the B-lineage and away from the T-lineage.

**HEBAlt imparts T-lineage potential to LK cells**

We previously showed that HEBAlt promoted T-cell development from LSK cells (22). To determine whether HEBAlt could rescue T-cell potential in LK cells, we retrovirally transduced E14.5 Lin− fetal liver cells with control MIGR1 constructs or HEBAlt-expressing constructs and cultured them overnight. GFP+ LK cells were sorted and placed in OP9-DL1 co-culture (Fig. 4). As previously observed, control-transduced LK cells did not become CD25+Thy1+ cells, and the total cell numbers were very low (Fig. 4C). Strikingly, however, LK cells transduced with HEBAlt differentiated into T-lineage progenitors, whereas cells transduced with HEBCan did not (Fig. 4A). We also transduced Lin− E14.5 fetal liver cells with HEBAlt or HEBCan, cultured them overnight and sorted GFP+ Lin− cells, which consist primarily of LK cells after overnight culture (Fig. 1A). These cells were placed in FTOC and analyzed after 7 days (Fig. 4B). Under these conditions, HEBAlt again enhanced T-cell potential, whereas HEBCan did not, both in terms of percentages and absolute numbers (Fig. 4C).

To compare the relative efficiency of LSK versus LK T-cell development in the presence of HEBAlt, we analyzed FTOCs at day 11 for the generation of cells expressing CD4 and CD8 (Supplementary Figure S2 is available at International Immunology Online). We also monitored expression of TCRγδ to assess whether HEBAlt affected the αβ/γδ T-cell

![Fig. 3.](https://academic.oup.com/intimm/article-abstract/22/12/963/701005)
lineage choice under these conditions. CD4+ or double positive (DP; CD4+CD8+) cells were not present at this time point, which was consistent with the normal time course of hanging drop FTOC (34). However, CD8+ immature single positive and/or TCRγδ+ cells were observed in the control-transduced and HEBAlt-transduced LSK-FTOCs as well as the HEBAlt-transduced LK-FTOCs. The control-transduced LK-FTOCs produced very few cells, as expected. Interestingly, there were higher percentages of CD8+ cells in the HEBAlt-transduced LK-FTOCs than in either of the LSK-FTOCs, and those cells developed into DP cells by day 14 of culture (data not shown). However, HEBAlt did not fully restore the cellularity of the LK-FTOC cultures (Supplementary Figure S2 is available at International Immunology Online and data not shown), indicating that other factors were still missing for a fully functional T-cell differentiation and proliferation program.

Notch signaling is sufficient to allow LK cells to enter the T-cell lineage

It is known that Notch1 can be up-regulated by E2A and HEB factors (35). Furthermore, we have observed that Notch1 is lower in LK cells than in LSK cells, suggesting that HEBAlt might restore T-cell potential to LK cells by enabling DL-Notch signaling. We tested this possibility by setting up OP9-GFP and OP9-DL1 co-cultures with LK cells that retrovirally expressed ICN (Fig. 5). ICN provides a strong Notch signal independently of ligand availability and thus is constitutively active. We analyzed the OP9-GFP co-cultures after 5 days for the presence of B-lineage (CD19+CD11b−) and myeloid (CD11b+CD19−) cells (Fig. 5A) versus T-lineage (CD25+Thy1+) cells (Fig. 5C). Control-transduced LK cells generated both B cells and myeloid cells on OP9-GFP stroma but very few T-lineage cells in either OP9-GFP or OP9-DL1 co-culture, as expected (Fig. 5C and D). However, ICN-transduced LK cells did produce T-lineage cells on OP9-GFP stroma and increased overall cell numbers, indicating that strong Notch signaling restored T-cell potential to at least some of the LK cells (Fig. 5C and E).

DL-Notch signaling inhibits B-cell development from early precursors but not from committed B-lineage cells (24). Interestingly, even though HEBAlt inhibited B-cell development from LK cells, exposure to DL or ICN alone did not (Fig. 5B). However, the combination of DL and ICN was sufficient to inhibit both B-cell and myeloid development. Moreover, the addition of ICN rescued T-cell potential in LK cells to an
even greater degree in the presence of endogenous DL than in its absence (Fig. 5D). The synergistic effect of ICN in addition to DL1 exposure suggests that ICN may have upregulated endogenous Notch1 expression within these cells, which would then be able to interact with DL ligands, consistent with the positive autoregulatory loop that has been previously described (35).

**HEBAlt regulates Ikaros, Notch1 and C/EBPα expression**

Since HEBAlt and Notch signaling could each independently restore T-cell potential to LK cells, we investigated the possibility that HEBAlt could up-regulate Notch1. LK cells include a mixture of precursors including B-lineage precursors, myeloid precursors and erythroid precursors (Supplementary Figure S1 is available at International Immunology Online; Figs 1 and 5)(32). Therefore, we generated B-lineage (AA4.1\(^+\)B220\(^-\)CD19\(^+\); Fig. 6A) and myeloid (CD11b\(^+\)c-kit\(^-\); Fig. 6D) precursors by culturing transduced sorted LSK cells on OP9-GFP stroma for 5 days. MIY-control and MIY-HEBAlt expressing B-lineage and myeloid cells were sorted and analyzed by Q-PCR for the expression of Notch1 and C/EBPα. Notch1 mRNA was low in pre-pro-B cells (Fig. 6B) and undetectable in myeloid cells (Fig. 6E), whereas C/EBPα was barely detectable in pre-pro-B cells (Fig. 6C). However, Notch1 mRNA was increased in both subsets in response to forced expression of HEBAlt. C/EBPα mRNA, by contrast, was decreased in HEBAlt-transduced myeloid cells (Fig. 6F).

To further investigate the ability of HEBAlt to modulate gene expression in early precursors, we grew transduced LSK cells in OP9-GFP co-culture for 4 days and then sorted out the CD45\(^+\)CD19\(^+\)lin\(^-\) populations (Fig. 7A). These cells are roughly equivalent to early pre-pro-B cells (33) and still retain some C/EBPα expression. Intriguingly, C/EBPα was again down-regulated in the presence of HEBAlt (Fig. 7B). We next examined the mRNA expression of Ikaros, which is thought to be a positive regulator of Notch1 expression and an indirect negative regulator of C/EBPα expression (11), and found that it was elevated in the presence of HEBAlt (Fig. 7C). Notch1 is higher in these HEBAlt-transduced cells as well (data not shown). The elevation of Ikaros and Notch1, coupled with the repression of C/EBPα in response to HEBAlt provides a partial mechanistic explanation for the ability of HEBAlt to restore T-cell potential to LK cells.
Discussion

Here, we show that HEBAlt can impart T-lineage potential to myeloid precursors and also show that it is capable of modulating the expression of key myeloid and lymphoid transcription factors. We therefore propose that HEBAlt, Notch1 and Ikaros participate in a positive cross-regulatory feedback loop to maintain Notch1 expression in early T-cell progenitors. Although other cross-regulatory loops have been described for B cell, macrophage and granulocyte commitment (12, 13), this is the first example of a feedback loop that may be operating during T-cell specification. Moreover, the critical myeloid factor C/EBPα is repressed in response to HEBAlt, suggesting that it may be important for inhibiting myeloid potential during early T-cell development. Importantly, our data demonstrate that HEBCan is unable to impart T-cell potential to LK cells, suggesting that HEBAlt plays a unique role in T-lineage specification. Interestingly, recent evidence suggests that HEB and E2A are bound to critical regulatory elements in the Notch1 promoter in situ and that disruption of this binding by Id3 leads to down-regulation of Notch1 at the β-selection checkpoint (35). It is therefore possible that HEBAlt collaborates with E47 to drive Notch1 expression and to repress C/EBPα during early T-cell development, but it remains to be seen whether regulation of Notch1, Ikaros and/or C/EBPα by HEBAlt is direct or indirect.

Understanding the regulation of Notch1 during early thymocyte development is relevant not only to normal T-cell development but also to T-cell lineage malignancies since Notch1 activation mutations and translocations account for >50% of T-cell acute lymphoblastic leukemias in humans (36). Moreover, deregulated expression of Notch3, which is a downstream target of Notch1, has also been associated with T-ALL (37). In other studies in our laboratory, we have generated transgenic mice expressing HEBAlt under the control of the lck-proximal promoter and have found that some of these mice develop T-cell acute lymphoblastic lymphoma (M. Braunstein and M. K. Anderson, manuscript in preparation). These results are consistent with a regulatory relationship between HEBAlt and Notch signaling factors and a possible role for HEBAlt in the genesis of T-ALL.

Fig. 6. HEBAlt-transduced precursors express higher levels of Notch1 than control-transduced precursors. E14.5 fetal liver cells were depleted of lineage-positive (CD19, Gr1, Ter119 and F4/80) cells by MACS, transduced with MIGR1-control or MIGR1-HEBAlt retrovirus, cultured overnight and sorted for the transduced LSK populations. After 5 days of OP9-GFP co-culture with 5 ng ml⁻¹ IL-7, SCF and Flt3L, cells were sorted as shown and analyzed by Q-PCR. (A) FACS profiles for sorted populations of transduced AA4.1⁺B220⁺ pre-pro-B cells. (B) Expression of Notch1 mRNA in transduced pre-pro-B cells. (C) Expression of C/EBPα mRNA in transduced pre-pro-B cells. (D) FACS profiles for sorted populations of transduced CD11b⁻c-kit⁻ myeloid cells. (E) Expression of Notch1 mRNA in transduced myeloid cells. (F) Expression of C/EBPα mRNA in transduced myeloid cells. Q-PCR values were normalized to β-actin and represent mean ± SD of triplicate readings.
Fig. 7. Modulation of C/EBPα and Ikaros in response to HEBAlt overexpression in CD45stemCD19- precursors. CD45stemCD19- cells correspond to early pre-pro-B cells. E14.5 liver cells were obtained by MACS depletion of cells expressing CD19, F4/80, Gr1 or Ter119 and transduced with MIGR1-control or MIGR1-HEBAlt retroviral constructs. Transduced cells were cultured overnight in 10 ng ml-1 IL-7, SCF and Flt3L to allow GFP expression and then GFP+ LSK cells were sorted and plated on OP9-GFP co-cultures for 4 days. GFP+CD45stemCD19- cells were sorted and subjected to Q-PCR. (A) FACS profile of sort for CD45stemCD19- cells from day 4 LSK OP9-GFP co-cultures. (B, C) Q-PCR measurements of (B) C/EBPα and (C) Ikaros mRNA in MIGR1-control transduced and MIGR1-HEBAlt GFP+CD45stem cells. Q-PCR values were normalized to β-actin and represent the mean ± SD of triplicate readings.

Notch factors are used in many different developmental processes throughout metazoan phylogeny, whereas T-cell development is restricted to vertebrates. Therefore, it is possible that HEBAlt was involved in recruiting Notch expression to T-cell developmental networks during evolution. Furthermore, HEBAlt is expressed widely outside the hematopoietic system and thus could partner with Notch in other systems. The importance of HEBAlt may have been previously overlooked due to the difficulties in studying HEBAlt+ mice, which usually die around birth. Moreover, HEBAlt mice lack both HEBAlt and HEBCan, which could obscure opposing developmental influences during T-cell development and elsewhere. E2-2Alt, which is homologous to HEBAlt, is also expressed in early thymocytes, albeit at low levels and could thus provide partial compensation for the loss of HEBAlt. Addressing these questions awaits the development of tools for specific depletion E2-2Alt, E2-2Can, HEBAlt and HEBCan in a cell-type- and developmental stage-specific manner.

We show here that HEBAlt and ICN collaborate to inhibit myeloid development even in cells that have already become highly primed toward the myeloid lineage. Interestingly, deletion of Notch1 in early thymocytes results in the development of myeloid cells in the thymus (38), which is consistent with a requirement for continued Notch signaling in T-cell precursors to suppress the myeloid fate choice. A recent fate-mapping experiment has challenged this relationship by showing that cells that had expressed IL-7Rα at some point in their development did not generate myeloid cells (39). However, the developmental progression that leads to ETP generation in the thymus does not require IL-7Rα expression (40), leaving open the possibility that ETPs and even early DN2 cells retain myeloid potential. Moreover, we have found that HEBAlt represses the myeloid potential of LSK cells in a DL-Notch-dependent manner (33). These results further suggest that HEBAlt can inhibit early regulators of the myeloid fate choice.

The mechanism by which HEBAlt inhibits myeloid development in the presence of DL-Notch signals has yet to be determined; however, our data suggest that C/EBPα is a prime candidate. PU.1 and C/EBPα are present in the earliest myeloid progenitors, whereas Egr1 and Gfi-1 are up-regulated downstream of these regulators during myeloid development (12). Gfi-1 can also be up-regulated by the lymphoid factor Ikaros and is required for T-cell development and is thus not a likely target of negative regulation by HEBAlt (13, 41). However, Egr factors are not normally induced in T-cell precursors until after pre-TCR signaling (42). Therefore, since Egr factors are up-regulated at the same time that HEBAlt is down-regulated, they are potential targets of repression by HEBAlt.

Ikaros and C/EBPα act antagonistically during myeloid and lymphoid development. Ikaros represses C/EBPα and induces Notch1 expression as part of the lymphoid priming program (11). Moreover, C/EBPα can collaborate with PU.1 to reprogram T-cell precursors or B-cell precursors to myeloid lineages (14, 15, 43). Intriguingly, the regulatory region of C/EBPα contains a site which is bound by HEB factors in vivo (44). However, additional experiments will be required to evaluate whether HEBAlt directly influences the expression of C/EBPα, Ikaros, Egr1 and/or Notch1 in developing T-cell precursors. Future experiments will focus on identifying targets of HEBAlt, determining the molecular basis for its specificity and understanding how these factors interact within the gene regulatory networks that control hematopoiesis.

Supplementary data
Supplementary data are available at International Immunology Online.

Funding
Canadian Institutes of Health Research (MOP82861, NIP79923); Leukemia Research Fund; Sunnybrook Research Institute to M.K.A.; Ontario Graduate Scholarship to M.B.

Acknowledgements
We are grateful to J. C. Zúñiga-Pflücker, M. Ratcliffe and J. Rast for critical comments and helpful discussions. We appreciate the technical assistance of Jie Min, Qianqian Xu and Mathew Chui. We also thank G. Knowles and A. Khandani for their sorting expertise and the Comparative Research Facility at SRI for excellent animal care. The authors declare that there are no conflicts of interest.

References
HEBAlt restores T-cell potential


