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A dose-dependent role for EBF1 in repressing non-B cell specific genes

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Summary

In the absence of EBF1, B cell development is arrested at an uncommitted progenitor stage that exhibits increased lineage potentials. Previously, we investigated the roles of EBF1 (*E*) and its DNA binding partner Runx1 (*R*) by evaluating B lymphopoiesis in single (E^{het} and R^{het}) and compound haploinsufficent (ER^{het}) mice. Here, we demonstrate that reduced *Ebf1* gene dosage results in the inappropriate expression of NK cell lineage-specific genes in B cell progenitors. Moreover, prolonged expression of *Ly6a*/Sca-1 suggested the maintenance of a relatively undifferentiated phenotype. These effects were exacerbated by reduced expression of Runx1 and occurred despite expression of Pax5. Repression of inappropriately expressed genes was restored in most pre-B and all immature B cells of ER^{het} mice. Enforced EBF1 expression repressed promiscuous transcription in pro-B cells of ER^{het} mice and in $Ebf1^{-/-}Pax5^{-/-}$ fetal liver cells. Together, our studies suggest that normal levels of EBF1 are critical for maintaining B cell identity by directing repression of non-B cell-specific genes.

Introduction

Lineage specification is the process by which cells acquire fates that will be retained in their descendant cells [1]. As one of the most important drivers of B lineage specification, EBF1 (EBF/O/E-1/COE1) is essential for B lineage specification and development [2, 3]. EBF1 can direct cells towards the B cell fate at the expense of other cell lineages and promotes B cell development in the absence of upstream regulators, including IL-7 and the transcription factors PU.1, Ikaros or E2A [4-10]. Recent evidence suggests that EBF1 controls B lymphopoiesis in at least two ways by: 1) activating genes that encode essential components of the B lineage-specific program and 2) reinforcing B lineage commitment (together with Pax5) by repressing genes of other hematopoietic programs [10-13].

Previously, we demonstrated the synergistic activation of early B cell-specific genes by EBF1 and its regulatory partner Runx1 [14]. To determine the importance of interactions between these two factors *in vivo*, we analyzed the phenotypes of *Ebf1* (E^{het}), *Runx1* (R^{het}), or compound (ER^{het}) haploinsufficient mice. B cell numbers were reduced significantly in bone marrow and spleens and the progression of B cell development was impeded in E^{het} and ER^{het} mice [15]. Here, we demonstrate that a significant fraction of E^{het} and ER^{het} pro-B

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(and some pre-B) cells express NK cell-associated genes, including *Cd244* (CD244/2B4), *Cd160* (CD160/BY55) and *Klrb1c* (NK1.1). Promiscuous expression of NK cell-specific genes was observed in the presence of Pax5; therefore, Pax5 expression alone is insufficient for the maintenance of B cell identity in the presence of reduced EBF1. Repression of NK cell-specific transcripts was restored by EBF1 in ER^{het} pro-B cells and $Ebf1^{-/-}Pax5^{-/-}$ fetal liver cells. Together, these data confirm the importance of EBF1 in B cell commitment.

Results and Discussion

Promiscuous expression of NK cell-specific genes in E^{het} and ER^{het} mice

To characterize differences between gene expression patterns of B cell progenitors from *WT* and ER^{het} mice we utilized Affymetrix DNA microarray analysis with $B220^+CD2^+mIgM^-$ pre-B cells as a source of mRNA. The results indicated that multiple genes, including many that are normally expressed in NK cells, were expressed in the cells from ER^{het} mice (Supplemental Table 1); however, the data may have been influenced by co-purified $B220^+CD2^+$ NK cell progenitors [16]. Therefore, we assessed bone marrow cells for the co-expression of NK cell-specific surface proteins and CD19, the B cell 'commitment marker' [17]. CD244 (2B4) is expressed on some multipotent progenitors and on mature and progenitor NK cells [18, 19]. Flow cytometry of bone marrow-derived cells of *WT* and R^{het} mice detected distinct $CD19^+$ B cell and $CD244^+$ NK cell populations as well as infrequent, diffuse $CD19^+CD244^+$ double-positive cells (Fig. 1A). In contrast, significant, well-defined populations of $CD19^+CD244^+$ cells were detected in E^{het} (5.9%) and ER^{het} (8.8%) mice, indicating the mixing of lineage markers.

We examined the extent of co-expression of CD19 and CD244 together with the stage-specific markers of B cell development CD117 and BP-1. As expected [15], percentages of CD19-gated $CD117^+$ pro-B cells were increased in E^{het} and ER^{het} mice relative to *WT* and R^{het} mice (Fig. 1B, upper panel). Moreover, CD19-gated $CD117^+CD244^+$ cells increased from 1.5% to 15.2% in *WT* versus ER^{het} bone marrow. BP-1 is a marker of late pro-B cells of normal mice [20]. CD19-gated $CD244^+BP\text{-}1^+$ cells were nearly undetectable (1.0%) in *WT* mice, but they were increased substantially in E^{het} (5.9%) and ER^{het} (9.6%) mice (Fig. 1B, lower panel). The presence of CD24 on $B220^+CD43^+$ bone marrow-derived cells is indicative of pro-B cells (Fr. B-C′) as well [20]. The expression of CD244 on $B220^+CD43^+$-gated cells increased from 2.2% in *WT* mice to 13.5% and 19.5% in E^{het} and ER^{het} mice, respectively (Fig. 1C, upper panel). CD244 expression on $B220^+CD43^-$-gated $B220^{lo}$ pre-B cells (Fr. D) from *WT* and R^{het} mice was minimal and was not detected as a defined population (Fig. 1C, lower panel). In contrast, $CD43^-$-gated $CD244^+B220^{lo}$ bone marrow populations resolved well in E^{het} and ER^{het} mice (4.0% and 7.5%, respectively), but expression of CD244 was less than that observed at the pro-B cell stage. In the bone marrow, CD25 is a marker of pre-B cells [21]. We demonstrated previously that CD19-gated $CD25^+$ pre-B cells were reduced greatly in E^{het} and ER^{het} bone marrow [15]. CD244 was not detected on CD19-gated $CD25^+$ cells in the mutant mice (Supporting Information Fig. 1). CD244 was virtually absent on $B220^+CD43^-$-gated $B220^{med}$ (Fr. E, immature B cells) and $B220^{hi}$ (Fr. F, mature B cells) cells from mice of all four genotypes (Fig.1C, lower panel). Moreover, CD244 was not detected on live-gated $mIgM^+$ bone marrow cells (Fig. 1D) or $mIgM^+$ splenocytes (Supporting Information Fig. 2A) of any of the mice. Similar results for Fr. B-F cells were obtained when the expression of NK1.1 was evaluated (Supporting Information Fig. 3). $NK1.1^+CD244^+$ NK cells were present at normal percentages in spleens of mice of each genotype (Supporting Information Fig. 2B). Taken together, CD244 expression is detected on early progenitors of E^{het} and ER^{het} mice up to and including some Fr. D pre-B cells, but CD244 is not detected on most Fr. D cells, $CD25^+$ pre-B cells or at later stages of B cell development.

Quantitation of NK cell-specific gene expression in developing B cells

CD244 is expressed on a subset of transiently reconstituting, multipotent hematopoietic progenitors, as well as NK cells [19]. Therefore, we evaluated whether genes activated at later stages of the NK cell program were expressed in B cell progenitors from the haploinsufficient mice. Quantities of specific transcripts in $CD117^+CD19^+$ (pro-B) and $CD117^-CD19^+mIg^-$ (late pro-B/pre-B) cells were analyzed using qRT-PCR. ER^{het} pro-B cells (Fig. 2A, upper panel) expressed increased *Cd244* transcripts relative to *WT* cells (5.2-fold; p=0.019). Interestingly, we detected similarly increased transcripts of the NK-specific *Cd160* gene (encoding CD160) when comparing *WT* and ER^{het} pro-B cells (5.9-fold; p=0.018). Transcripts of *Klrb1c* (encoding NK1.1) were increased 2.6-fold in pro-B cells from ER^{het} bone marrow (p<0.0001). Significantly elevated *Cd244*, *Cd160*, and *Klrb1c* transcripts were detected in late pro-B/pre-B cells of ER^{het} versus *WT* mice (Fig. 2A, lower panel). *Klrb1c* transcripts were increased in E^{het} (4.7-fold; p=0.099) and ER^{het} (9.6-fold; p=0.0063) cells relative to those of *WT* (and R^{het}) mice. The increases in these transcripts are largely due to BP-1^+ B cell progenitors, which are present in similar numbers in mice with the four genotypes [15].

Promiscuous expression of NK cell-specific genes in E^{het} and ER^{het} mice suggests that transcription factors required to activate their expression may be up-regulated in the haploinsuficient B cell progenitors. To address this question, we performed qRT-PCR to test whether transcripts of the *Id2* and *Nfil3* (E4BP4) genes, which have been linked with the generation of mature NK cells in vivo [22, 23], are increased in the mutant B cells. Significant changes in *Id2* transcripts were not detected in any $CD117^+CD19^+$ cells from mice of the four genotypes (data not shown). However, *Nfil3* transcripts were significantly increased in $CD117^+CD19^+$ cells of E^{het} and ER^{het} mice (3.9-fold, p=0.041 and 5.8-fold, p=0.036, respectively; Fig. 2B). Significant differences in these transcripts were not observed between pre-B cells of the four genotypes. Thus, promiscuous expression of *Nfil3*/E4BP4 may contribute to the expression of NK cell specific genes in the mutant mice.

Simultaneous expression of CD19 and NK cell transcripts in B cells occurs in the presence of Pax5

The expression of NK cell markers on $CD19^+$ B cells and co-expression of B and NK cell transcripts suggests that signals necessary for B lineage commitment are decreased in the haploinsufficent cells. Expression of the *Cd19* and *Cd79a* (encoding Igα) genes is dependent critically on the B lineage commitment factor Pax5 [24-26]. Previously, we detected relatively normal amounts of *Pax5* transcripts in populations of pro-B and pre-B cells of E^{het} and ER^{het} mice [15]. This suggests that the presence of NK cell transcripts in early B cells is not due to the silencing of *Pax5* genes, but it is dependent on sufficient dosage of EBF1.

To demonstrate the co-expression of B and NK cell-specific transcripts in single cells, we employed multiplex RT-PCR analysis on purified $CD117^+CD19^+CD244^+$ bone marrow cells from ER^{het} mice. All cells expressed *Hprt*, which was amplified as a ubiquitously expressed control (data not shown). Forty-one percent of cells were positive for *Cd244* transcripts. The percentage of cells expressing *Cd244* was less than expected, which may have been due to low levels of transcripts in many of the sorted cells. Importantly, of the cells that expressed *Cd244* transcripts, 95% (38/40) expressed transcripts of the B cell commitment factor Pax5 and 100% (40/40) expressed the BCR signaling component *Cd79a* (Fig. 2C). Additionally, *Cd160* and *Klrb1c* transcripts were present in 20% (8/40) and 17.5% (7/40) of cells, respectively. These data clearly indicate the co-expression of B cell and NK cell programs in committed $CD19^+$ pro-B cells when EBF1 expression is reduced. Furthermore, these data suggest that EBF1 functions similarly to, and perhaps in conjunction

with *Sfpi-1*/PU.1, which represses expression of NK cell-specific genes including *Cd244*, *Klra2* and *Klrb1b* in a dose-dependent manner [27].

Ly6a/Sca-1 expression is increased in pro-B/pre-B cells with reduced EBF1 expression

To evaluate the possibility that cells from ER^{het} mice transitioned incompletely to 'committed' $CD19^+$ B cells, we assessed expression of *Ly6a* (encoding Sca-1), a marker of hematopoietic progenitors [28]. When compared to pro-B cells of *WT* mice, *Ly6a* transcripts were increased significantly in R^{het}, E^{het} and ER^{het} pro-B cells (2.7-, 4.2- and 7.7-fold, respectively; p=0.0002 for ER^{het}) (Fig. 2D, upper panel). Furthermore, *Ly6a* transcripts were increased 5.1-fold (p=0.029) compared to levels in *WT* late pro-/pre-B cells (Fig. 2D, lower panel). When evaluated by flow cytometry, expression of Sca-1 was increased dramatically on pro-B cells (Fr. B-C′) from the mutant mice (Fig. 2E). The Sca-1^+CD24^+ population of $B220^+CD43^+$-gated bone marrow cells increased 6.7-fold from *WT* to ER^{het} mice (8% to 54%, respectively). This population was increased to a lesser extent in the single mutants (22% for R^{het} and 32% for E^{het}). These data further confirm that the haploinsufficient cells are less differentiated than developing B cells from *WT* mice.

Increased expression of EBF1 reduces NK cell-specific transcripts

Our data suggested that the partial loss of B cell identity in E^{het} and ER^{het} cells is largely due to *Ebf1* haploinsufficiency. To address this hypothesis, ER^{het} pro-B cells were infected with retroviruses to express control GFP or EBF1(18-591) as separate proteins. Purified GFP^+ cells were analyzed for expression of specific transcripts using qRT-PCR (Fig. 3A). Enforced expression of EBF1 increased B cell-specific *Igll1* (λ5) transcripts 5-fold (p=0.065). In contrast, *Cd244* and *Cd160* transcripts were reduced significantly by EBF1 (70% and 80%, respectively; p=0.0014 and <0.0001, respectively). We conclude that expression of EBF1 is sufficient to repress NK cell-specific genes in ER^{het} pro-B cells. Previously, $Ebf1^{-/-}$ $B220^+$IL-$7R^+$ progenitors were shown to have NK, myeloid and T cell lineage potential; however, enforced expression of EBF1 repressed myeloid cell-specific genes and prevented alternate fates. The ability of EBF1 to repress non-B cell fate decisions was linked with repression of fate-promoting transcription factors including *Sfpi1*, *Cebpa*, *Id2* and *Id3* [10,11].

To confirm that the reduced expression of NK lineage genes by EBF1 does not require Pax5, we reconstituted $Ebf1^{-/-}Pax5^{-/-}$ fetal liver cells with EBF1 (Fig. 3B). Quantities of *Igll1* transcripts in $B220^+$ $Ebf1^{-/-}Pax5^{-/-}$ fetal liver cells expressing EBF1(18-429) increased 309-fold (p<0.0001) compared to transcripts in control $B220^+$ cells. *Cd244* and *Cd160* transcripts were reduced 69% and 58%, respectively (p=0.0282 and 0.02154, respectively) in the presence of EBF1. Together, these data demonstrate the ability of EBF1 to repress genes that are expressed promiscuously in B cells of ER^{het} mice. Notably, this activity is observed in the absence of Pax5.

Concluding Remarks

Here, we demonstrated that markers of NK and undifferentiated cells are expressed promiscuously on bone marrow-derived pro-B cells, but not on $CD25^+$ pre-B, $mIgM^+$ B cells or peripheral B cells of E^{het} and ER^{het} mice. The data suggest a partial loss of B cell identity that is due largely to *Ebf1* haploinsufficiency and is exacerbated by *Runx1* haploinsufficiency. This is of particular interest because the expression of most genes does not change due to haploinsufficiency [29]. The synergy between *Ebf1* and *Runx1* genes in ER^{het} mice is highly significant because compound effects were not observed in $Pax5^{+/-}Runx1^{+/-}$, $Ebf1^{+/-}Ikzf1^{+/-}$ or $Ebf1^{+/-}Gfi1^{+/-}$ mice (K.L. and J.H., and H. Xu and H. Singh, unpublished data). Furthermore, enforced expression of EBF1 in ER^{het} pro-B cells

increased the expression of B lineage-specific genes while repressing expression of NK-specific genes. Similar results were obtained in *Ebf1*$^{-/-}$*Pax5*$^{-/-}$ fetal liver cells suggesting that EBF1 may repress genes to enforce B lineage commitment in the absence of Pax5. These observations, together with our detection of *Pax5* transcripts in single *ER*^{het} cells that expressed NK cell transcripts, suggest that EBF1 contributes directly to B lineage commitment. This hypothesis is strengthened further by the loss of NK lineage potential in common lymphoid progenitors that express EBF1 [30]. The data do not detract from the seminal importance of Pax5 in the regulation of B lineage commitment [17,26], but they advocate strongly that the two factors regulate commitment together.

Materials and Methods

Mice

Ebf1$^{+/-}$ mice and *Runx1*$^{+/-}$ mice were described previously [15]. All experiments were approved by the Institutional Animal Care and Use Committee at National Jewish Health.

Antibodies and flow cytometry

Cell staining, analysis and sorting were performed as described previously [15] with the following antibody additions: CD24-biotin (BD Pharmingen, San Diego, CA, USA) with SA-APC-Cy7 (Biolegend, San Diego, CA, USA) for detection, CD244-Alexa Fluor 647 (eBioscience, San Diego, CA, USA) and Sca-1(Ly6a/e)-PE and CD244.2-PE (BD-Pharmingen, San Jose, CA, USA).

RNA isolation and quantitative RT-PCR (qRT-PCR)

RNA isolation, preparation of cDNA and qRT-PCR for detection of transcripts were performed as described [15]. Data are reported as mean±SEM. For single cell PCR, individual CD117^{+}CD19^{+}CD244^{+} bone marrow cells were sorted into wells of 96 well plates using a MoFlo XDP (Beckman Coulter, Brea, CA). Cells were quick frozen and processed prior to detection of indicated transcripts or control *Hprt* transcripts as described [30]. Primers used in these studies are listed in Supporting Information Table 2.

Preparation of recombinant retroviruses, transduction of cells and analysis

Retroviruses for expression of EBF were described previously (14). Bone marrow cells were isolated as described without depletion (15). *ER*^{het} pro-B cells were expanded in 10% complete IMDM containing 5 ng/mL IL-7 and Flt-3L (R&D Systems, Minneapolis, MN). On days 5 and 6, cells were transduced by spinfection at 2000 rpm at 25°C for 2 hours with retroviral supernatants containing 4 μg/mL polybrene (Sigma, St. Louis, MO) and 10 mM HEPES, pH7.4. Following spinfection, cells were returned to expansion media. Transduced cells were purified on day 4 using a MoFlo XDP (Beckman Coulter). *Ebf1*$^{-/-}$*Pax5*$^{-/-}$ fetal liver cells (14) were transduced by a single spinfection with 10 μg/mL polybrene. At 96 hours, transduced, B220^{+} cells were purified as above and RNA was isolated as described previously [15].

Statistical analysis

Data shown were pooled from at least three experiments. Two-tailed, unpaired Student *t*-tests were calculated with Prism (Graphpad Software, Inc, La Jolla, CA). Values of $p < 0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COE-1	Collier-Olf-EBF
EBF1	Early B cell factor 1
Hprt	hypoxanthine/guanine phosphoribosyl transferase
Klrb1c	killer cell lectin-like receptor, subfamily A, member 7
***Ly6a*/Sca-1**	Lymphocyte antigen 6, complex A/stem cell antigen 1
mIgM	membrane IgM
NK1.1	Natural Killer antigen 1.1

O/E-1	Olf-EBF-1
WT	wild type
E^{het}	*Ebf1*$^{+/-}$
R^{het}	*Runx1*$^{+/-}$
ER^{het}	*Ebf1*$^{+/-}$*Runx1*$^{+/-}$
***Sfpi1-1*/PU.1**	*Spleen focus forming virus (SFFV) proviral integration oncogene*/Purine-rich box-1
E2A	E12 and E47 factors encoded by *Tcfe2a*
Nfil3/E4BP4	Nuclear factor, interleukin 3-regulated/E4 promoter-binding protein 4
Id2	inhibitor of DNA binding 2

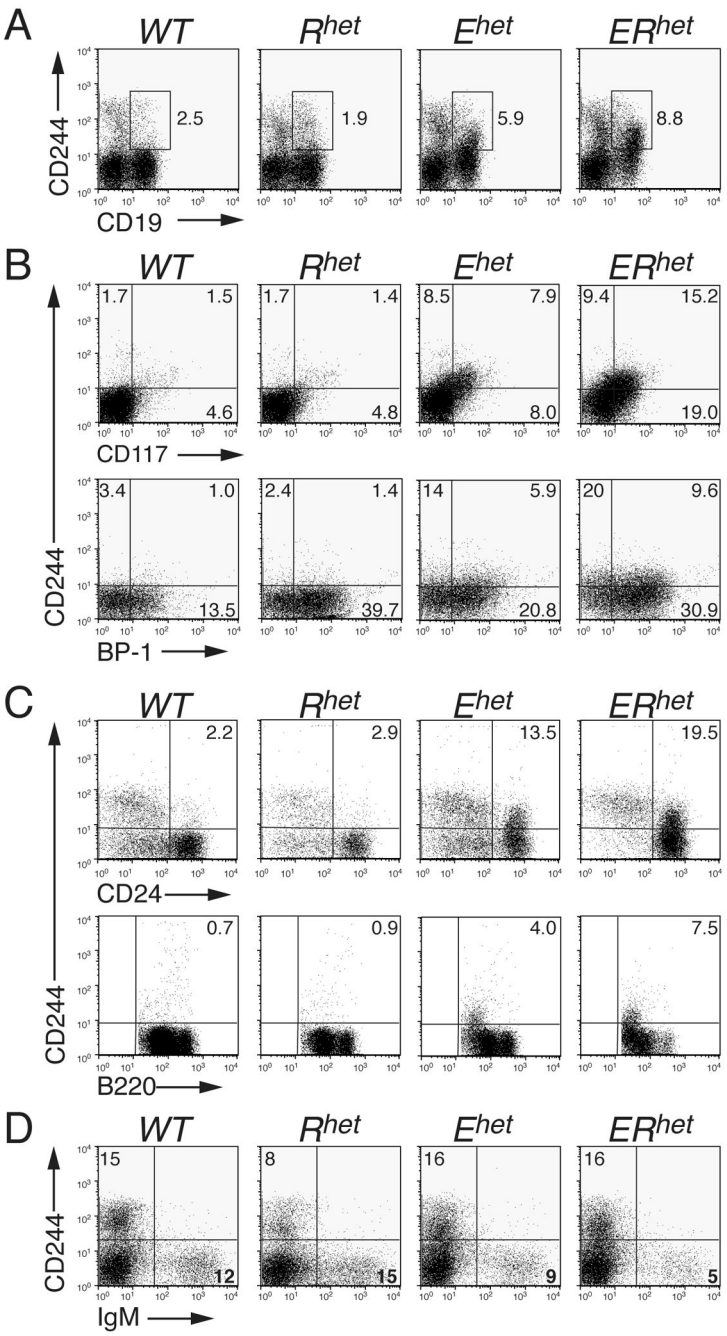

FIGURE 1.

Loss of B cell identity in haploinsufficient mice. (A) Detection of the NK cell marker CD244 (2B4) and the B cell commitment marker CD19 together on haploinsufficent bone marrow cells. (B) Detection of CD244 on various stages of developing B cells in the bone marrow. Cells were pre-gated on $CD19^+$ cells. (C) Expression of CD244 on $B220^+CD43^+$-gated $CD24^+$ bone marrow cells (upper panel); expression of CD244 on $B220^+CD43^-$-gated $B220^{lo}$ bone marrow cells (lower panel). (D) Lack of CD244 expression on $CD19^+$-gated $mIgM^+$ cells from the bone marrow. All data are representative of greater than four independent experiments.

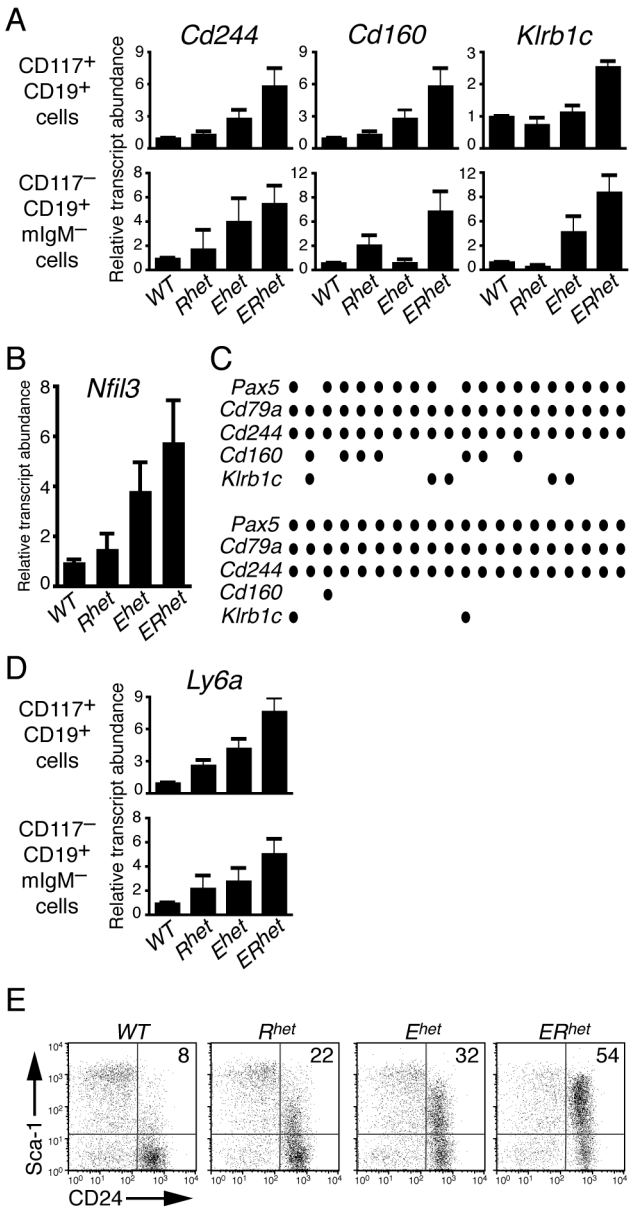

FIGURE 2.

Quantitation of NK cell-, B cell- and progenitor-specific transcripts in bone marrow-derived B cells of haploinsufficient mice. All transcript levels were normalized to *Hprt* transcripts (mean±SEM). qT-PCR data are representative of three or more independent experiments and three to six mice per genotype. (A) qRT-PCR analysis of NK cell-specific transcripts in pro-B (upper panel) and late pro-B/pre-B cells (lower panel). (B) qRT-PCR analysis of *Nfil3* transcripts in $CD117^+CD19^+$ bone marrow cells. (C) Single-cell multiplex amplification of cDNA derived from individual $CD117^+CD19^+CD244^+$ bone marrow cells. Circles indicate positive signals for each RT-PCR probe set. (D) qRT-PCR analysis of *Ly6a* transcripts in bone marrow cell populations. (E) Expression of Sca-1 on $B220^+CD43^+$–gated $CD24^+$ bone marrow cells. The data are representative of two independent experiments.

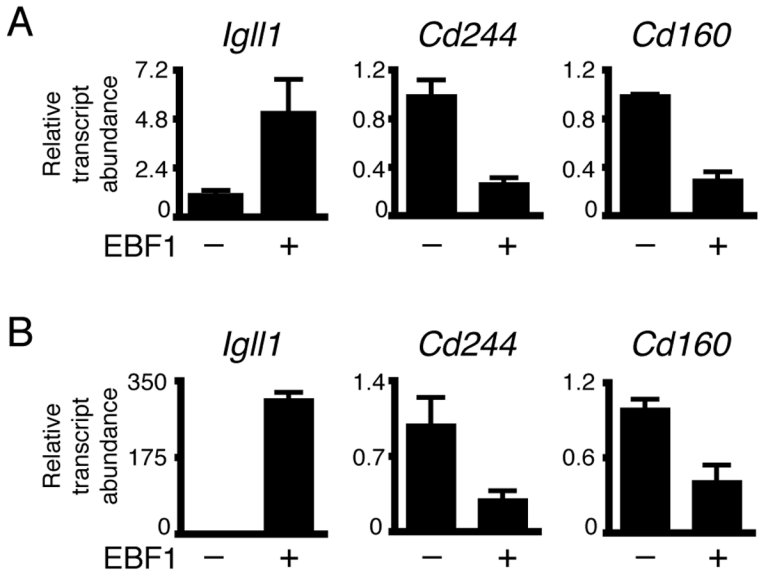

FIGURE 3.

Enforced expression of EBF1 increases B cell-specific and decreases NK cell-specific transcripts in pro-B cells of ER^{het} mice and $Ebf1^{-/-}Pax5^{-/-}$ fetal liver cells. (A) qRT-PCR analysis of pro-B cells of ER^{het} mice expressing control GFP or EBF1(18-591)(co-expressed with GFP). (B) qRT-PCR analysis of $Ebf1^{-/-}Pax5^{-/-}$ fetal liver cells expressing EBF1(18-429) and GFP. $B220^{+}$ uninfected cells were analyzed as a control. All transcripts were normalized to *Hprt* transcripts (mean±SEM). Data are from three independent experiments.