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Normal Hemopoiesis and Lymphopoiesis in the Combined Absence of Numb and Numblike

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The mammalian ortholog of the conserved Drosophila adaptor protein Numb (Nb) and its homolog Numblike (Nbl) modulate neuronal cell fate determination at least in part by antagonizing Notch signaling. Because the Notch pathway has been implicated in regulating hemopoietic stem cell self-renewal and T cell fate specification in mammals, we investigated the role of Nb and Nbl in hemopoiesis using conditional gene targeting. Surprisingly simultaneous deletion of both Nb and Nbl in murine bone marrow precursors did not affect the ability of stem cells to self-renew or to give rise to differentiated myeloid or lymphoid progeny, even under competitive conditions in mixed chimeras. Furthermore, T cell fate specification and intrathymic T cell development were unaffected in the combined absence of Nb and Nbl. Collectively our data indicate that the Nb family of adaptor proteins is dispensable for hemopoiesis and lymphopoiesis in mice, despite their proposed role in neuronal stem cell development. The Journal of Immunology, 2007, 178: 6746–6751.

The adaptin protein Numb (Nb) is a membrane-associated evolutionarily conserved adaptin protein that regulates cell fate determination in Drosophila via its ability to antagonize Notch signaling (1, 2). In mice, Nb and its homolog Numblike (Nbl) seem to play redundant roles in the maintenance of several types of neuronal stem cells during fetal development (3, 4), raising the possibility that Nb family members may play a role in stem cell self-renewal or lineage commitment in other tissues.

Stem cells are required for homeostatic maintenance and repair of regenerating tissues such as the skin, the intestine, and the hemopoietic system. Adult hemopoietic stem cells (HSCs) are principally located in the bone marrow (BM) and have the unique capacity to both self-renew and differentiate into all mature blood cell lineages, thus providing life-long reconstitution of the adult hemopoietic system. All HSCs are found within the lineage-negative (Lin−) fraction of the BM, and can be further identified as expressing high levels of the c-Kit receptor (CD117) and stem cell Ag-1 (Sca-1) (reviewed in Refs. 5, 6). Thus, they have been termed LSK (Lin− Sca1+ c-Kit+) cells. Because the Notch signaling pathway has been implicated in HSC self-renewal (7, 8), it is possible that antagonism of Notch signaling via Nb family members might interfere with HSC homeostasis.

Notch signaling has also been shown to be crucial for the development of T cells in the thymus, as in the absence of Notch1 or RBPJ (a downstream signaling component of all Notch receptors) in early T progenitors, T cell development is completely abrogated (9, 10). Early thymic precursors expressing high levels of CD117 (c-KitR) that have entered the thymus from the BM give rise to several sequential stages of T cell development (11, 12). These subsets can be distinguished by their differential expression of the surface markers CD44 and CD25. The most immature thymocyte, termed double-negative (DN1) (for CD4− CD8−), is characterized by the expression of high levels of CD44 and the absence of CD25. DN1 thymocytes give rise to the DN2 subset (CD44+ CD25−), then DN3 (CD44+ CD25+) and DN4 (CD44+ CD25+). Subsequently DN4 cells differentiate to the CD4+ CD8+ double-positive stage that is the precursor of functional CD4+ or CD8+ mature T cells.

In addition to its role in early T cell fate specification, Notch1 is also necessary for the transition of DN3 cells to the double-positive stage (10, 13), but is dispensable thereafter (14).

To determine directly whether Nb or Nbl play a role in either hemopoiesis or lymphopoiesis, we have used Cre-lox technology to generate mutant mice in which Nb and Nbl can be simultaneously inactivated in BM HSCs. Surprisingly we find that Nb and Nbl are dispensable for hemopoiesis, even in differentiated lineages (such as T lymphocytes) where Notch signaling plays a critical role.

Materials and Methods

Generation of a loxP-flanked Nb gene

To obtain the conditional Nbllox allele, the first three exons of Nbl that encode the functionally essential phosphoryrosine-binding domain were flanked by loxP sites. To this end, contiguous genomic fragments encompassing the first six Nblox exons were subcloned from the genomic phase clones E53.11 and E53.12, which were isolated from a genomic library by hybridization of radioactively labeled Nblox cDNA (clone C33-1/C53-4). The genomic library was generated from 129SveV isogenic DNA partially digested with MboI, shotgun-ligated into BamHI-digested calf intestinal alkaline phosphatase-treated arms of the bacteriophage vector λ DASH II.

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1 Abbreviations used in this paper: Nb, Numb; Nbl, Numblike; BM, bone marrow; dKO, double knockout; DN, double negative; Lin−, lineage-negative; LSK, Lin− Sca1+ c-Kit+; CMP, common myeloid precursor; CLP, common lymphoid precursor; HSC, hemopoietic stem cell; pIpC, polyinosinic-polycytidylic.

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that were in vitro packaged with Gigapack XL (Stratagene). Appropriate genomic Nb restriction fragments were subcloned into the NotI, Asci, and PmeI sites of the targeting vector FSTV1, a second generation vector of TNLOX1-3 (15); a 1.8-kb Hind III/EcoRI 5′ fragment as the homology arm placed upstream of the 5′ loxP site, a 4.0-kb EcoRI/XhoI fragment as the deletion arm encompassing the first three coding exons, and a 4.1-kb XhoI/EcoRI 3′ fragment as the homology arm placed downstream of the 3′ loxP site and adjacent to the negative selection gene HSV-TK.

To screen progeny, PCR primers P3 (nblfla/22) 5′-GGTCTCGGTAGCTTAACCTCTATTACC-3′ and P4 (nbl3a/L24) 5′-GGCAGCGAGTTAAGAAACATCTCTCTC-3′, yielding products of ~280 bp (Nb+) and ~320 bp (Nblox/lox) were used. Progeny from later generations derived from chimeric founder mice were mated to Fip-deleter mice (16) to eliminate the PKGkneo selection cassette by recombination of the FRT sites. Deletion of the loxP-flanked Nb exons by Cre-mediated recombination was detected by PCR using the primers P5 (nbl5a/L24) 5′-CAGTCTGGACACCTGCTTC-3′ and P4, yielding a product of ~350 bp specific for deletion of the three targeted exons (Nblox/lox). Mice conditionally targeted for Nb and Nbl are available from The Jackson Laboratory (stock number 5348; www.jax.org/index.html) and RIKEN (BRC nos. 01267 and 01268; http://brc.riken.jp/lab/animal/eurl/).

Generation of mice double mutant for Nb and Nbl

Mice homozygous for the Nblox/lox allele were intercrossed to Mx-Cre: Nblox/lox mice (15, 17) to produce Mx-Cre:Nblox/loxNbllox/lox (double knockout (dkO)) and Nblox/lox:Nbllox/lox (control) littersmates. Cre expression for deletion of the loxP-flanked Nb exons was induced by ip injection of 450 μg of polyinosinic-polycytidylic (pIpc) five times every 4 days as described previously (9). Deletion efficiency of Nb and Nbl was assessed by PCR on genomic DNA extracted from various hemopoietic tissues. This study has been reviewed and approved by the Service Vétérinaire de l’Etat de Vaud, Switzerland.

PCR to assess expression of Nb and Nbl

RNA was prepared from total BM, Lin− BM, or thymocytes using TRIzol. Lin− BM was prepared by depletion of total BM cell suspension with a mix of FITC-labeled rat mAbs including CD3 (17A2), CD4 (GK1.5), CD8α (55-67), CD11b (M1/70), B220 (RA3-6B2), Gr1 (RB6-8C5), CD161 (NK1.1), and Ter119 together with anti-Rat Ig M450 Dynabeads (Dynal Biotech) by standard protocols. Purity of depletion was assessed by FACS analysis of the resultant population and was >90%. Semiquantitative RT-PCR was performed on 3-fold dilutions of cDNA. PCR primers were as follows: Nb 5′-GGGATTTCTCCTGCTTGAGC-3′ and 5′-GGATCGACT TCAGAAGGAGGTG-3′ yielding products of 200 and 350 bp as they span the alternatively spliced exon 8; Nbl 5′-CTGAAAAAACCTCAGAG GGAG-3′ and 5′-CACAGGCAACACCTCAGAGGA-3′ yielding a 260-bp product (18); and β-actin 5′-GGTGGGCCGCTAGCCAA-3′ and 5′-CTTCGTTGATGCAGCAAGCTTC-3′ yielding a 450-bp fragment.

BM chimeras

CD45.1+ B6-SJL mice were purchased from The Jackson Laboratory. Competitive BM chimeras were set up as described previously (9). Briefly, 1.5 × 10^7 T cell-depleted Mx-Cre:Nblox/loxNbllox/lox or Nblox/loxNbllox/lox BM (both CD45.2+) were transferred together with 1.5 × 10^7 wild-type CD45.1+ BM into lethally irradiated (1000 rad) CD45.1+ recipient mice. Six weeks after BM transfer deletion of Nb and Nbl was initiated by injection of plpC as described previously. For population turnover studies, mice were injected with BrdU as described previously (14). BM chimeras were analyzed at 3 or 10 mo postdeletion or 3 mo after a primary or secondary transfer.

Monoclonal Abs and flow cytometry

Single-cell suspensions of BM and thymus were prepared and stained following standard protocols for flow cytometry using mAbs conjugates previously described in our laboratory (FITC, biotin, and Alexa Fluor 647) as described (9, 19, 20), or purchased (PE, PE-Cy5, and allophycocyanin) from eBioscience. The Alexa Fluor 647 conjugation kit was purchased from Molecular Probes. BrdU uptake studies were as previously described (14), using the BrdU staining kit (BD Biosciences). All samples were analyzed on a four-color FACS Calibur (BD Biosciences) or the six-color FACS Canto (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences). FACS sorting was performed on a FACS Aria (BD Biosciences). Dead cells were gated out by their forward and side scatter profile.

Results

Nb and Nbl are expressed in hemopoietic tissues

Nb and Nbl have distinct but overlapping expression patterns both in the adult and during embryogenesis (21). As the Nb homolog Nbl is able to substitute for Nb in neuronal precursors (3), it is conceivable that they may also play redundant roles in other developing organs such as the hemopoietic system. Consistent with this hypothesis, Nb and Nbl have been shown to be expressed (by RT-PCR or Northern or Western blots) in hemopoietic tissues such as the thymus, spleen, lymph nodes, and PBLs in both mouse and human models (18, 22–25). To determine whether Nb and Nbl are also expressed in the most immature hemopoietic cells, we performed semiquantitative RT-PCR on RNA isolated from BM from which all hemopoietic lineage cells originate (Fig. 1A). Although both Nb and Nbl are expressed in thymocytes as expected from previous published results (18, 24, 25) (Fig. 1A), only Nb is detectable in total BM (Fig. 1A). However after enrichment of BM for HSCs and early progenitors by depletion of the lineage-positive fraction as shown in Fig. 1B (upper), Nbl can also be detected in the remaining Lin− fraction (Fig. 1A). Although the Lin− subset (around 3% of total BM) is highly enriched for LSK-HSC activity, it is still quite heterogeneous and also comprises common myeloid precursors (CMPs, defined as CD117^Sca1^−) and common lymphoid precursors (CLPs, defined as CD117^lowSca1^−CD127^−). Therefore we further fractionated the Lin− subset by FACS sorting (Fig. 1B, lower), into LSK-HSCs, CMPs, and CLPs. As shown in Fig. 1C, both Nb and Nbl are expressed in each of these stem cell populations.
subsets, thus providing support for a possible role for one or both of these genes in the hemopoietic system.

**Generation of mice deficient for both Nb and Nbl in the BM**

Although conventional KO of Nbl are viable (O. Zilian, unpublished observation) (4), Nb KO are embryonic lethal at embryonic day 11.5 (15, 26). Therefore, to explore the effects of loss of Nb and Nbl on adult hemopoiesis, we used the Cre-loxP system combined with the Mx-Cre transgene (17) to conditionally delete Nb alone or together with Nbl.

Homozygous Nblox/lox mice previously generated in this laboratory (15) were intercrossed to Mx-Cre transgenic mice (17) to produce Mx-Cre:Nblox/lox (Nb KO) and Nblox/lox (Control) littermates. Conditional deletion of Nb alone in the BM showed no difference in any hemopoietic lineage (data not shown), possibly due to the previously documented redundancy of Nbl in tissues where both genes are expressed, such as in the developing brain (21, 24) and lymphoid tissues (18, 24, 25) (Fig. 1). As Nb has been shown to be able to replace Nbl in tissues (4), Nb KO are embryonic lethal at embryonic day 11.5 (15, 26). Therefore, to explore the effects of loss of Nb and Nbl simultaneously in postnatal or adult mice revealed no overt phenotype or any obvious hemopoietic abnormality or difference in cellularity compared with control Mx-Cre-negative mice up to 6 mo posttreatment with pIpC (data not shown). To rigorously determine whether Nb/Nbl double-deficient (dKO) BM precursor cells differed from control BM precursors under competitive conditions, mixed BM chimeras were generated. A 1:1 mixture of dKO Mx-Cre:Nblox/loxNbllox/lox (CD45.1<sup>+</sup>) and wild-type (CD45.1<sup>+</sup>) or control Nblox/loxNbllox/lox (CD45.2<sup>+</sup>) and wild-type (CD45.1<sup>+</sup>) BM were transferred into lethally irradiated CD45.1<sup>+</sup> hosts. Four weeks after transfer, chimerism was confirmed among PBLs (Fig. 3), and mice were subsequently treated with pIpC to activate Mx-Cre-mediated deletion of Nb and Nbl. No differences in chimerism of either lymphoid or myeloid cells in the blood were observed 4 mo postdeletion (Fig. 3), nor in any other hemopoietic or lymphoid organ (data not shown). Notably, no differences were observed in the total cellularity of chimeric BM, spleen and thymus between dKO and control mice (data not shown). A PCR strategy was used to assess the deletion efficiency in the BM isolated from these mixed chimeras 10 mo postdeletion.
FIGURE 3. Analysis of BM hemopoiesis in mice simultaneously lacking Nb and Nbl. A, Chimerism in PBLs in mixed BM chimeras pre- and postdeletion. The percentage of CD45.2+ donor experimental (□) or control BM (□) was determined in lymphoid (CD3+ and B220+) or myeloid (CD11b+ and Gr1+) cells in PBLs isolated from mixed BM chimeras 4 wk posttransfer (predeletion) and 4 mo postdeletion with pIpC. B, Deletion of Nb and Nbl in BM and thymocytes isolated from mixed BM chimeras. PCR on DNA isolated from BM (left column) and thymocytes (right column) taken from individual competitive BM chimeras 10 mo postdeletion. In the dKO chimeras (Mx-Cre: Nblox/lox Nblox/lox), a 700-bp fragment corresponding to the deleted Nb allele was observed (lanes 1–4, top row), together with a 350-bp fragment derived from wild-type competitor BM (lanes 1–4, second row). No 400-bp fragment indicative of the unrecombined Nb floxed allele was detected in these mice (lanes 1–4, second row) showing that deletion of the Nb allele was highly efficient in both BM and thymus. In lanes 5–7 (second row), the presence of both the Nb floxed allele (derived from control Nblox/lox Nblox/lox) and wild-type allele (competitor BM) were observed as expected. No deletion was observed in the absence of the Mx-Cre transgene. Similarly, deletion of the Nbl allele was assessed by PCR in the bottom two rows. The results show efficient deletion of Nbl in lanes 1–4 corresponding to BM and thymocytes derived from the dKO chimeras (Mx-Cre: Nblox/lox Nblox/lox), whereas no deletion of Nbl was observed in the control BM (lanes 5–7). C, Histograms showing the number of donor-derived (CD45.2+) cells in BM subsets from control (□) or dKO (□) competitive chimeras. Gran, granulocytes (CD11b+ Gr1+); Mac, macrophages (CD11b+ Gr1+); Megas, megakaryocytes (CD41+). B, B220+ B cells. Results are mean ± SD of three to five mice of each genotype 3 mo postdeletion. No significant differences were observed between control and dKO-derived BM cells by Student's t test (p > 0.05) except for B cells (p = 0.02). D, Representative FACS plots showing Sca-1 vs c-Kit (CD117) staining of Lin− CD45.2+ BM 3 or 10 mo postdeletion (left panels). The percentage of LSK cells in each plot is indicated. The absolute number of donor-derived (CD45.2+) cells in BM subsets from control (□) or dKO (□) competitive chimeras (Mx-Cre: Nblox/lox Nblox/lox, Nblox/lox Nblox/lox) is shown (right panels). Control chimeras (□) and dKO chimeras (□) are indicated. Results are mean ± SD of 6 mice of each genotype. No significant differences were observed between control and dKO-derived BM cells by Student's t test (3 mo, p = 0.7; 10 mo, p = 0.05). E, 2nd BM transfer was performed 3 mo postdeletion and analyzed 3 mo later. The absolute number of donor-derived (CD45.2+) LSK-HSCs, CLPs (Lin− CD127+ CD117low Sca1low), and CMPs (Lin− CD117+ Sca1+) in BM at indicated times after deletion is shown. Control chimeras (□) and dKO chimeras (□) are shown. Results are mean ± SD of 6 mice of each genotype. No significant differences were observed between control and dKO-derived BM cells by Student’s t test (p > 0.2). F, Turnover of donor-derived (CD45.2+).
As shown in Fig. 3B, the floxed Nb or Nbl alleles were undetectable in BM cells isolated from dKO chimeras, whereas PCR products indicative of the deleted alleles were clearly detectable.

To assess the contribution of Nb/Nbl-deficient BM to the hematopoietic system under competitive conditions, FACS analysis was performed on donor-derived CD45.2 BM isolated from dKO or control mixed chimeras 3 mo postdeletion. No significant differences in the number or relative distribution of myeloid, erythroid, or lymphoid subpopulations were observed in the absence of both Nb and Nbl except for a minor increase in B cells (Fig. 3C).

Because Nb and Nbl redundantly control the self-renewal of neuronal stem cells (3) we also quantitated HSCs in dKO and control chimeras. Long-term reconstituting HSC activity is exclusively found in the small subset of LSK-HSCs principally found in the BM (reviewed in Ref. 5). No significant difference in the proportion or number of LSK-HSCs was observed in the dKO chimeras compared with control chimeras at 3 or 10 mo postdeletion (Fig. 3D). Furthermore, no differences in the number of other immature progenitors such as CLPs and CMPs were observed at any time studied (Fig. 3D).

Moreover, 3 mo (Fig. 3E) or 10 mo (data not shown) after a secondary transfer of BM isolated from these competitive chimeras, the proportion and number of LSK-HSCs, CLPs, and CMPs were similar in both dKO and control chimeras indicating that the absence of both Nb and Nbl does not confer any competitive advantage or disadvantage to long-term reconstituting HSCs.

In an independent set of competitive chimeras, the turnover rate of LSK-HSCs, CLPs, and CMPs was determined by BrdU labeling. No difference in the percentage of donor-derived BrdU+ (cycling) cells was observed between dKO and control chimeras (Fig. 3F). Taken together these data indicate that both self-renewal and differentiation of HSCs can occur normally in the combined absence of Nb and Nbl.

Thymocytes develop normally in the combined absence of Nb and Nbl

To determine unequivocally whether Nb or Nbl activity plays a role in intrathymic development or T cell lineage commitment, we analyzed thymocytes from dKO or control chimeras 3 mo after deletion of Nb and Nbl. The same PCR strategy was used to verify efficient deletion of both Nb and Nbl floxed alleles in thymocytes derived from dKO BM (Fig. 3B). Phenotypic analysis of the principal thymus subsets defined by staining with CD4 and CD8 (Fig. 4A) showed similar frequency and absolute number (Fig. 4B) of each subset derived from dKO or control donor BM cells. Moreover, when the immature DN population was further subdivided based on the differential expression of CD44 and CD25, the relative frequency (Fig. 4C) and absolute number of each DN subset (DN1–4) was unchanged in Nb/Nbl-deficient chimeras compared with control chimeras (Fig. 4D). Finally, the early thymic precursor subset of DN1 thymocytes (CD117+CD44+CD25+) that is the earliest intrathymic precursor known to be responsive to Notch signaling (11, 12) was also not affected in the combined absence of Nb and Nbl (Fig. 4D). Collectively these data indicate that neither Nb nor Nbl play an essential role in thymocyte development or intrathymic lineage commitment.

LSK-HSCs, CLPs, and CMPs 3 mo postdeletion. The percentage of BrdU+ cells was determined in each immature BM subset after 18-h treatment with BrdU. Control (□) and dKO (●) are shown. Results are mean ± SD of 10 mice of each genotype. No significant differences were observed between control and dKO-derived BM cells by Student’s t test (p ≥ 0.09).

FIGURE 4. Normal reconstitution of thymocyte subsets in dKO mixed BM chimeras. A, Representative FACS plots of CD8 vs CD4 on donor-derived (CD45.2+) thymocytes 3 mo postdeletion. The percentage of the subset is indicated in each quadrant. B, The absolute number of donor-derived (CD45.2+) cells in major thymus subsets defined by CD4 vs CD8 expression as DN (CD4+CD8−), double positive (CD4+CD8+), CD8 single positive (CD4+CD8−), and CD4 single positive (CD4+CD8+). Control (□) and dKO (●) are shown. Results are mean ± SD of six mice at 3 mo postdeletion. No significant differences were observed between control and dKO-derived BM cells by Student’s t test (p ≥ 0.25). C, Representative FACS plots of immature thymocyte subsets as defined by staining with CD25 vs CD44 on donor derived (CD45.2+) DN thymocytes 3 mo postdeletion. The percentage of the subset is indicated in each quadrant. D, The absolute number of donor-derived (CD45.2+) thymocytes in early thymic precursors (CD117+CD44+CD25−), DN1 (CD25−CD44+), DN2 (CD25−CD44+), DN3 (CD25−CD44+), and DN4 (CD25−CD44+). Control (□) and dKO (●) are shown. Results are mean ± SD of four to six mice 3 mo postdeletion. No significant differences were observed between control and dKO-derived BM cells by Student’s t test (p ≥ 0.12).
Discussion
The possibility that Nb may play a role in T cell development or lineage commitment has previously been investigated by both gain- and loss-of-function approaches. Overexpression of an Nb transgene specifically in thymocytes failed to influence T cell development, even under competitive conditions (25). Similarly conditional deletion of a floxed allele of Nb during thymus development had no obvious phenotype (18). However, because the lck proximal promoter that is first active in DN3 thymocytes was used to overexpress (25) or delete (18) Nb in both these experimental systems, it is possible that any effects occurred too late to impact on T cell development. Alternatively, as both Nb and Nbl are expressed in the thymus (18, 23–25), the function of Nb in the T cell lineage could have been compensated by Nbl, as is the case for neuronal stem cells (3).

The data presented in this study confirm and extend these earlier studies by demonstrating directly that all stages of T cell development occur normally in the combined absence of Nb and Nbl. Using an Mx-Cre transgene to simultaneously delete floxed alleles of both Nb and Nbl in BM precursor cells, we show that both the percentage and the absolute number of all major thymocyte subsets are unaffected by Nb/Nbl double deficiency, even when tested under rigorous conditions in mixed BM chimeras.

Although our data clearly establish that Nb family members do not play an essential role in T lymphopoiesis, they do not directly address the issue of whether Nb and/or Nbl antagonist Notch signaling in this lineage. In this regard, overexpression of an Nb transgene in thymocytes led to a modest reduction in expression of certain Notch target genes such as Hes1 and pTα (25), although no impairment of T cell development was observed. Similarly, Nb/ Nbl double deficiency might result in up-regulation of expression of Notch target genes without affecting T cell lineage commitment or maturation. Alternatively, the antagonistic function of Nb or Nbl on Notch signaling may be species- or tissue-specific.

In conclusion, our data provide genetic evidence that both Nb and Nbl are dispensable for hemopoiesis and lymphopoiesis in the adult mouse. Despite the fact that Nb plays an important role in neuronal cell fate specification in Drosophila via its ability to antagonize Notch signaling (1, 2), we were unable to detect any effect of Nb/Nbl double deficiency in the mouse hemopoietic system, even in processes (such as HSC self-renewal and T cell lineage commitment) in which Notch signaling plays a critical role (7, 28).

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