

# Impairment of Natural Killer Cytotoxic Activity and Interferon $\gamma$ Production in CCAAT/Enhancer Binding Protein $\gamma$ -deficient Mice

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## Summary

We have investigated in vivo roles of CCAAT/enhancer binding protein  $\gamma$  (C/EBP $\gamma$ ) by gene targeting. C/EBP $\gamma$ -deficient (C/EBP $\gamma^{-/-}$ ) mice showed a high mortality rate within 48 h after birth. To analyze the roles of C/EBP $\gamma$  in lymphoid lineage cells, bone marrow chimeras were established. C/EBP $\gamma^{-/-}$  chimeras showed normal T and B cell development. However, cytolytic functions of their splenic natural killer (NK) cells after stimulation with cytokines such as interleukin (IL)-12, IL-18, and IL-2 were significantly reduced as compared with those of control chimera NK cells. In addition, the ability of C/EBP $\gamma^{-/-}$  chimera splenocytes to produce interferon (IFN)- $\gamma$  in response to IL-12 and/or IL-18 was markedly impaired. NK cells could be generated in vitro with normal surface marker expression in the presence of IL-15 from C/EBP $\gamma^{-/-}$  newborn spleen cells. However, they also showed lower cytotoxic activity and IFN- $\gamma$  production when stimulated with IL-12 plus IL-18 than control NK cells, as observed in C/EBP $\gamma^{-/-}$  chimera splenocytes. In conclusion, our study reveals that C/EBP $\gamma$  is a critical transcription factor involved in the functional maturation of NK cells.

Key words: gene targeting • natural killer cells • C/EBP $\gamma$  • interleukin 15 • interferon  $\gamma$

The CCAAT/enhancer binding proteins (C/EBPs)<sup>1</sup> belong to a family of basic leucine zipper transcription factors (1, 2). So far six members are isolated and share well conserved COOH-terminal regions, which contain a basic region for DNA binding and a leucine zipper motif for dimerization. Gene targeting revealed that each member plays a critical role in cellular proliferation and differentiation in various tissues. For example, nuclear factor (NF)-IL6, also designated C/EBP $\beta$ , is essential for macrophage function (3, 4) and involved in adipocyte differentiation, working coordinately with C/EBP $\delta$  (5). C/EBP $\alpha$  is essential for hepatocyte function and adipocyte differentiation (6, 7).

Furthermore, C/EBP $\epsilon$ , expressed specifically in myeloid lineage cells, plays a critical role in granulopoiesis (8).

C/EBP $\gamma$  is a member of the C/EBP family. C/EBP $\gamma$  can bind to the  $\mu$ EBP-E binding sites (E sites) in both the IgH enhancer and the Vh1 promotor and was originally identified as an Ig enhancer binding protein, Ig/EBP-1 (9, 10). C/EBP $\gamma$  can also bind to the regulatory elements for other genes such as G-CSF and IL-4 (11, 12). Although other members are expressed in a relatively tissue-specific or inducible manner, expression of C/EBP $\gamma$  is ubiquitous and constitutive (9). In addition, C/EBP $\gamma$  does not contain transcription activating domains. It can nevertheless interact with other transcription factors through the leucine zipper domain and function as a dominant negative form (10). In some cases, C/EBP $\gamma$  can augment the DNA binding ability of other transcription factors (12). Thus, C/EBP $\gamma$  is considered to be a regulator for transcription factors with

<sup>1</sup>Abbreviations used in this paper: AP, activator protein; C/EBPs, CCAAT/enhancer binding proteins; IRF, IFN regulatory factor; LT, lymphotoxin; NF, nuclear factor; RAG, recombination activating gene; RT, reverse transcriptase; STAT, signal transducer and activator of transcription.

the leucine zipper domain. However, the biological roles of C/EBP $\gamma$  remain unclear.

To clarify *in vivo* functions of C/EBP $\gamma$ , we have generated C/EBP $\gamma$ -deficient (C/EBP $\gamma^{-/-}$ ) mice by gene targeting. C/EBP $\gamma^{-/-}$  mice showed a high mortality rate within 48 h after birth, indicating that neonatal survival requires C/EBP $\gamma$ . By establishing bone marrow chimeras, we found that C/EBP $\gamma$  is critical for NK cell functions. NK cells are large granular lymphocytes derived from the bone marrow and essential for innate immunity (13, 14). NK cells can mediate cytotoxicity against tumor cells and virus-infected cells and produce immunomodulatory cytokines such as IFN- $\gamma$ . These activities were impaired in C/EBP $\gamma^{-/-}$  NK cells, establishing that C/EBP $\gamma$  is a novel regulator for functional NK cell maturation.

## Materials and Methods

**Generation of C/EBP $\gamma^{-/-}$  Mice.** A BALB/c-derived genomic fragment including the C/EBP $\gamma$  gene was provided by Dr. S. Nagata (Osaka University, Osaka, Japan). As the long arm of the homology region, an 8.8-kb genomic fragment including the first exon, the first intron, and part of the second exon was used. The neomycin resistance gene derived from pMC1Neo-poly(A) (15) was inserted into the targeting vector to disrupt the basic DNA binding and leucine zipper regions. A short arm of the homology region was amplified by PCR. The MC1 herpes simplex virus thymidine kinase (16) was inserted 5' upstream of the homology region.

E14-1 embryonic stem cells, which were derived from 129/SvJ (129) mice, were transfected with a linearized targeting vector by electroporation. Homologous recombinants were identified among double-resistant clones against G418 and gancyclovir by PCR and Southern blot analysis. Generation of chimeras and mutant mice was essentially as described (17).

**Reverse Transcriptase-PCR for C/EBP $\gamma$ .** Splenic B and T cells were purified from B6 spleen cells using Magnetic Cell Sorter (MACS<sup>®</sup>; Miltenyi Biotec) with B220 and CD3 microbeads, respectively. For purification of NK cells, DX5<sup>+</sup> cells were first prepared from B6 spleen cells using MACS<sup>®</sup> with biotinylated DX5 and streptavidin microbeads. Then the cells were stained with anti-CD3 and anti-DX5. CD3<sup>-</sup>DX5<sup>+</sup> cells were sorted with EPICS Elite (Coulter Immunology) and used as NK cells for reverse transcriptase (RT)-PCR. Total RNAs were extracted from tissues or cells using Sepazol-RNA I (Nacalai Tesque). Oligo-dT-primed reverse transcription was performed with Super-script-RT (GIBCO BRL). Then cDNAs were subjected to PCR analysis. Primers for C/EBP $\gamma$  were as follows: sense primer, 5'-GGCCGCTCGGAGTGGAGGCCGTCTGGG-3'; antisense primer, 5'-ACGTTGTCTGCGAGGCTGTGCGCATGC-3'. The length of the amplified product was 547 bp. These primers cannot amplify the genomic DNA because sense and antisense primers are located in the first and second exons, respectively. Cycling conditions were 35 cycles of 94°C for 30 s, 65°C for 30 s, and 75°C for 1 min. Primers for  $\beta$ -actin were as follows: sense primer, 5'-CCCACACTGTGCCATCTAC-3'; antisense primer, 5'-TACGGATGTCAACGTCACAC-3'. Cycling conditions were 24 cycles of 94°C for 15 s and 60°C for 30 s.

**Generation of Radiation Chimeras.** The C/EBP $\gamma$  genotype of neonates obtained by intercrossing heterozygous mutant mice was determined by PCR. The splenocytes were taken from the

neonates within 12–18 h after birth and used as sources for hematopoietic stem cells. They were injected intravenously into recombination activating gene (RAG)2<sup>-/-</sup> B6 mice (18) that had received 12 Gy from an X-ray irradiation system, MBR-1520R (Hitachi Medical Corp.), before transfer. The recipient mice were given 1 mg/ml neomycin sulfate and 1,000 U/ml polymixin B in their drinking water after irradiation and analyzed 6–10 wk after reconstitution. To evaluate the chimerism in NK cells, we used surface marker Ly9.1, the allele of which is carried by 129 but not B6 mice, as used by Ogasawara et al. (19). The genetic background of the donors in our experiments was a mixture of 129 and B6. Therefore, chimeras, of which the donor was Ly9.1<sup>+</sup>, were used for our experiments. In all reconstituted mice used, >99% of CD3<sup>-</sup>DX5<sup>+</sup> cells were positive for Ly9.1 expression (data not shown).

**Flow Cytometric Analysis.** Single-cell suspensions from thymus, spleen, or cultured cells were incubated with anti-CD16/32 (PharMingen) to minimize nonspecific staining. They were then stained with cocktails of mAbs conjugated to FITC, PE, biotin, or Cy-Chrome for 20 min at 4°C. The biotinylated Abs were developed with streptavidin conjugated to PE or Cy-Chrome (PharMingen). All mAbs, with the exception of PE-labeled anti-IgD (Southern Biotechnology Associates), were purchased from PharMingen. Flow cytometric analysis was performed using a FACSCalibur<sup>™</sup> with CELLQuest<sup>™</sup> software (Becton Dickinson).

**Proliferation Assay.** Fresh splenocytes (10<sup>5</sup> cells per well) were cultured in complete RPMI 1640 (RPMI 1640 medium supplemented with 10% FCS, 2-ME, penicillin, and streptomycin) with 10  $\mu$ g/ml anti-IgM (Zymed Labs.) plus 0.5  $\mu$ g/ml anti-CD40 (PharMingen), 10  $\mu$ g/ml LPS (055:B5; Sigma Chemical Co.), 20 ng/ml IL-2 (Genzyme Corp.), 0.1  $\mu$ g/ml anti-CD3 (PharMingen) plus 0.1  $\mu$ g/ml anti-CD28 (PharMingen), and 2.5  $\mu$ g/ml Con A (Sigma Chemical Co.) in 96-well plates. 48 h later, they were pulsed with 0.2  $\mu$ Ci of [<sup>3</sup>H]thymidine (NEN Research Products) and cultured for a further 15 h.

**Analysis of NK Cell Activity.** Splenocytes from bone marrow chimeras were incubated with 2 ng/ml IL-12, 20 ng/ml IL-18, and 2 ng/ml IL-12 plus 20 ng/ml IL-18 or 500 U/ml IL-2 for 24 h, and their cytotoxic activities against YAC-1 cells were measured as described previously (20, 21). Spontaneous cytotoxic activity was measured by incubating splenocytes with <sup>51</sup>Cr-labeled YAC-1 cells in the absence of cytokines for 4 h.

**ELISA and RT-PCR for IFN- $\gamma$ .** Splenic cells at 10<sup>5</sup> cells per well in 96-well plates were cultured in complete RPMI 1640 in the presence or absence of 2 ng/ml IL-12 and/or 20 ng/ml IL-18 for 24 h. Amounts of IFN- $\gamma$  in harvested supernatants were measured by ELISA using DuoSet (Genzyme Corp.) according to the manufacturer's instructions. The lowest detection limit of ELISA is 10 pg/ml. Semiquantitative RT-PCR for IFN- $\gamma$  was performed as described previously (22).

**In Vitro Culture of Newborn Spleen Cells.** Newborn spleen cells were cultured in complete RPMI 1640 with 300 ng/ml IL-15 (Genzyme Corp.) at 10<sup>6</sup> cells per well in 24-well plates. 10 d later, cells were harvested, washed four times, and used for further analysis. For cytotoxic activities, harvested cells were incubated with labeled YAC-1 cells in the absence of cytokines for 4 h. Their cytotoxic activities against YAC-1 cells were then measured as described previously (20). For IFN- $\gamma$  measurement, harvested cells were cultured at 10<sup>5</sup> cells per well in 96-well plates with 2 ng/ml IL-12 and 20 ng/ml IL-18 for a further 24 h. Then, amounts of IFN- $\gamma$  in the culture supernatants were measured with ELISA.

**RT-PCR Analysis of Cultured Spleen Cells.** Total RNAs were purified from 10-d-cultured newborn spleen cells with IL-15, re-

verse transcribed, and amplified. Primers and amplifying conditions for IL-12R $\beta$ 1, IL-12R $\beta$ 2, and IL-18R were described previously (21).

**Western Blot Analysis of Cultured Spleen Cells.** Newborn spleen cells cultured with IL-15 for 10 d were stimulated with 2 ng/ml IL-12 or 20 ng/ml IL-18 for 20 min and lysed in buffer containing 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, 10 mM NaF, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, and 10 mM  $\beta$ -glycerophosphate. For signal transducer and activator of transcription (STAT)4 activation, the cell lysates were immunoprecipitated with anti-STAT4 Ab (Santa Cruz Biotechnology) and Protein A-Sepharose (Amersham Pharmacia Biotech). The immunoprecipitates were separated on SDS-PAGE, transferred onto a nitrocellulose membrane, and blotted with antiphosphotyrosine 4G10 (Upstate Biotechnology Inc.) or anti-STAT4 Ab. For c-Jun NH<sub>2</sub>-terminal kinase (JNK) activation, the cell lysates were separated on SDS-PAGE, followed by blotting with antiphospho-JNK Ab (Promega Corp.) or anti-JNK1 Ab (Santa Cruz Biotechnology). Proteins bound to the Abs were visualized using the enhanced chemiluminescence system (Dupont).

## Results and Discussion

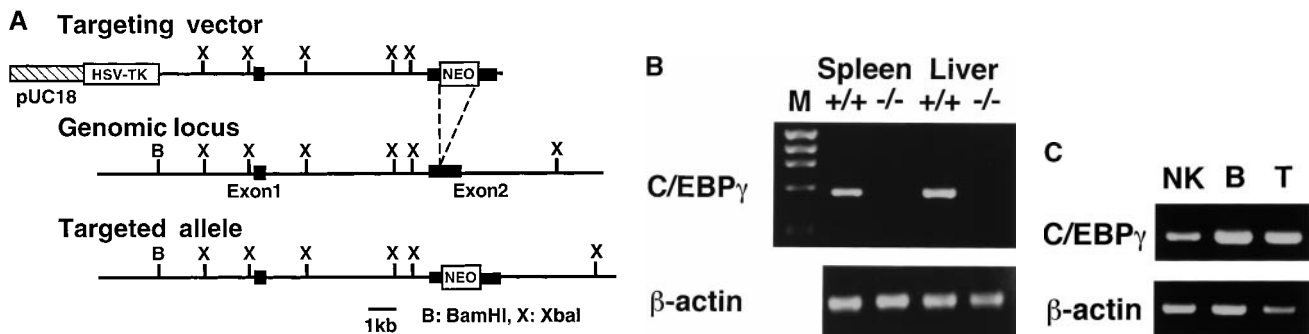
**Generation of *C/EBP $\gamma$* <sup>-/-</sup> Mice.** The *C/EBP $\gamma$*  genomic locus was disrupted by inserting the neomycin resistance gene into the second exon (Fig. 1 A). This insertion resulted in disruption of the basic and leucine zipper domains, which are essential for DNA binding and dimer formation. Homologous recombinants were obtained through a double selection with G418 and gancyclovir. Targeted clones were injected into B6 blastocysts to generate chimeric mice, which were bred to achieve germline transmission. By mating heterozygous mutants, homozygous mutants were obtained at a frequency of the expected Mendelian ratio (22.6% of littermates). *C/EBP $\gamma$*  expression was detected in wild-type newborn spleens and livers, but not in those of homozygous mutants as expected (Fig. 1 B). General appearance at birth was indistinguishable among *C/EBP $\gamma$* <sup>+/+</sup>, *C/EBP $\gamma$* <sup>+/-</sup>, and *C/EBP $\gamma$* <sup>-/-</sup> mice. However, only 11% of

**Table I.** Postnatal Survival of *C/EBP $\gamma$* <sup>-/-</sup> Mice

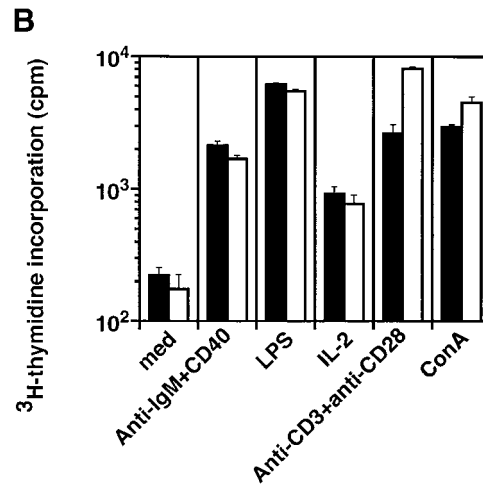
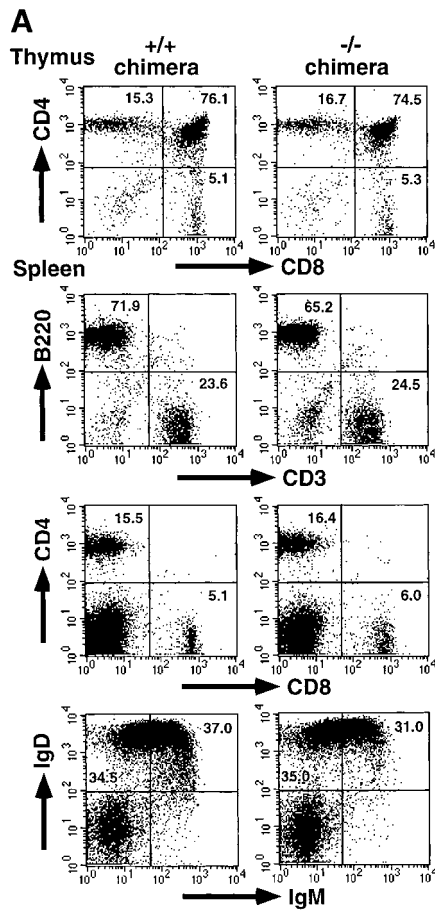
Time	Alive (%)	No. dead
<i>h</i>		
At birth	18 (100)	0
0–12	18 (100)	0
12–24	12 (66.7)	6
24–36	9 (50.0)	3
36–48	3 (16.7)	6
48–60	2 (11.1)	1
>60	2 (11.1)	0

homozygous mutants could survive >60 h, although they were healthy until 12 h after they were born (Table I). These results indicate that *C/EBP $\gamma$*  is involved in early neonatal survival but not embryonic survival. By histological examination, *C/EBP $\gamma$* <sup>-/-</sup> mice at 24 h showed emphysematous changes in their lungs (data not shown). However, it is not clear at the time of this writing whether the lung lesions alone can account for the high mortality of homozygous mutants at the early neonatal stage.

***C/EBP $\gamma$*  Deficiency Does Not Affect T or B Cell Development.** *C/EBP $\gamma$*  is expressed in T and B cells (9). Furthermore, NK cells, which belong to a distinct lymphoid lineage from T and B cells (13, 14), also expressed *C/EBP $\gamma$*  mRNA (Fig. 1 C). To investigate in vivo roles of *C/EBP $\gamma$*  in these lymphoid lineage cells, we generated bone marrow chimeric mice by transferring newborn spleen cells into lethally irradiated, RAG2<sup>-/-</sup> B6 mice. Thymocytes from *C/EBP $\gamma$* <sup>-/-</sup> chimeras showed normal T cell development (Fig. 2 A). Splenocytes from *C/EBP $\gamma$* <sup>-/-</sup> chimeras also showed normal population of CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> T cells (Fig. 2 A). Furthermore, analysis of surface markers such as B220, IgM, and IgD revealed that B cell maturation was not disturbed in *C/EBP $\gamma$* <sup>-/-</sup> chimeras (Fig. 2 A). In



**Figure 1.** Generation of *C/EBP $\gamma$* <sup>-/-</sup> mice and expression of *C/EBP $\gamma$*  mRNA. (A) Schematic representation of the *C/EBP $\gamma$*  targeting vector, the *C/EBP $\gamma$*  genomic locus, and the targeted *C/EBP $\gamma$*  allele. The targeting vector contains the herpes simplex virus thymidine kinase (HSV-TK) gene 5' upstream of the long arm homology region and the neomycin resistance gene (NEO) in the middle of the second exon. (B) RT-PCR analysis of newborn spleen and liver RNAs from *C/EBP $\gamma$* <sup>+/+</sup> and *C/EBP $\gamma$* <sup>-/-</sup> mice. Total RNAs were isolated and analyzed for *C/EBP $\gamma$*  and  $\beta$ -actin expression by RT-PCR. M,  $\Phi$ X174/HaeIII digest marker. (C) RT-PCR analysis for *C/EBP $\gamma$*  in lymphoid lineage cells. Total RNAs were isolated from splenic CD3<sup>-</sup>DX5<sup>+</sup> (NK), B220<sup>+</sup> (B), and CD3<sup>+</sup> (T) cells and analyzed for *C/EBP $\gamma$*  and  $\beta$ -actin expression by RT-PCR.



**Figure 2.** Development and function of T and B cells in  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  chimeras. (A) Flow cytometric analysis of thymus and spleen from  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  chimeras. Single-cell suspensions from the thymus and spleen were stained with indicated Abs and analyzed using FACSCalibur™ with CELLQuest™ software. The percentages of the quadrants are shown. (B)  $C/EBP\gamma^{+/+}$  (filled columns) and  $C/EBP\gamma^{-/-}$  (open columns) chimeras splenocytes were cultured with indicated mitogens or Abs and analyzed for their proliferation by measuring [<sup>3</sup>H]thymidine incorporation. Five independent experiments were performed with similar results. The data indicate mean  $\pm$  SD of triplicate samples of one representative experiment.

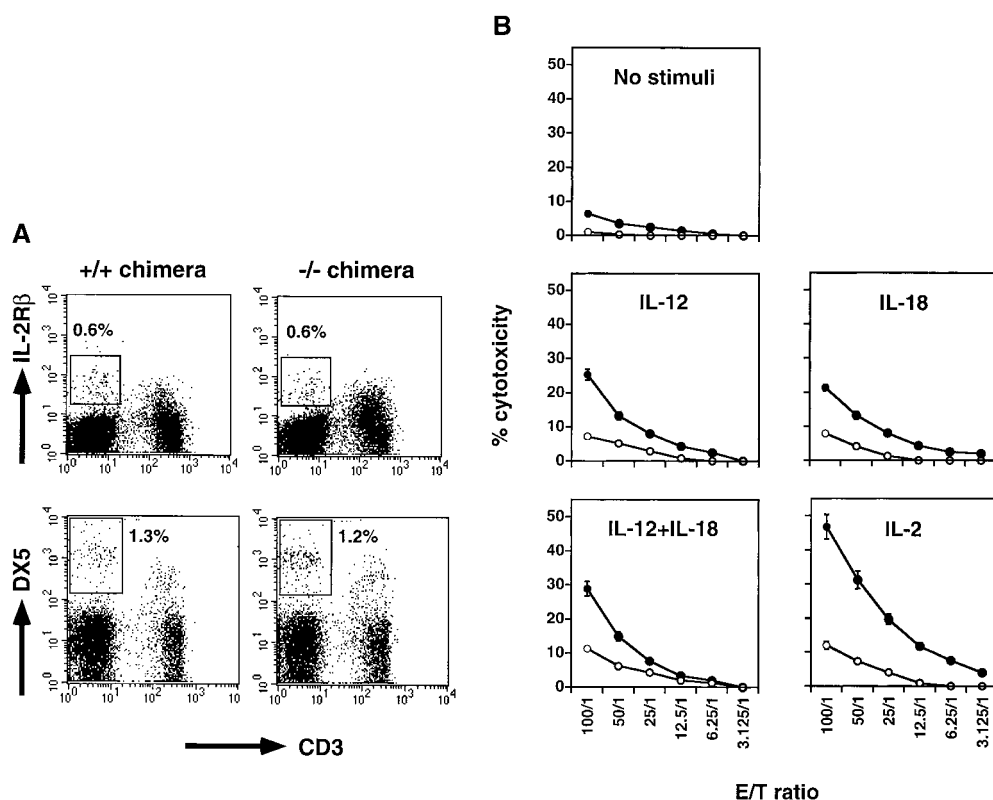
addition to surface phenotype, proliferative responses to mitogens or stimulating Abs were not significantly different between  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  chimeras (Fig. 2 B). Taken together, these results suggest that  $C/EBP\gamma$  is not essential for functional T and B cell development.

**Impaired Splenic NK Cell Activity in  $C/EBP\gamma^{-/-}$  Chimeras.** NK cells can be identified as  $CD3^{-}IL-2R\beta^{+}$ ,  $CD3^{-}NK1.1^{+}$ , or  $CD3^{-}DX5^{+}$  cells by flow cytometry. However, the NK1.1 analysis in the chimeras is limited, because only some mice in a mixed genetic background of 129 and B6 carried the NK1.1 allele, of which expression is detected on B6 but not 129 NK cells. Therefore, the NK cell population was analyzed for  $IL-2R\beta$  and  $DX5$  expression. Splenic  $CD3^{-}IL-2R\beta^{+}$  cells in  $C/EBP\gamma^{-/-}$  chimeras were detected at levels equivalent to those in  $C/EBP\gamma^{+/+}$  chimeras (Fig. 3 A). Furthermore, the frequency of  $CD3^{-}DX5^{+}$  cells was also comparable between  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  chimeras (Fig. 3 A). Percentages of  $CD3^{-}DX5^{+}$  cells were larger than those of  $CD3^{-}IL-2R\beta^{+}$  cells in both control and  $C/EBP\gamma^{-/-}$  chimeras splenocytes, as described previously (23).

Next, NK cytotoxicity was measured by YAC-1 cell-killing activity in the absence or presence of various cytokines (Fig. 3 B). Spontaneous cytotoxicity of  $C/EBP\gamma^{-/-}$  chimeras splenocytes (1.0% at 100:1) was impaired as compared with that of control chimeras splenocytes (6.4% at 100:1; Fig. 3 B).  $IL-12$  and/or  $IL-18$  act on NK cells and can en-

hance their cytotoxic activity (21). When stimulated with these cytokines,  $C/EBP\gamma^{-/-}$  chimeras splenocytes also showed impaired killing activity as compared with control splenocytes (Fig. 3 B).  $IL-2$  is another stimulatory cytokine for NK cell activity (24). Decreased killing activity of  $C/EBP\gamma^{-/-}$  chimeras was also observed in the presence of  $IL-2$  (Fig. 3 B). Poly (I:C)-stimulated  $C/EBP\gamma^{-/-}$  chimeras splenocytes also showed lower cytotoxic activity than control splenocytes (data not shown).

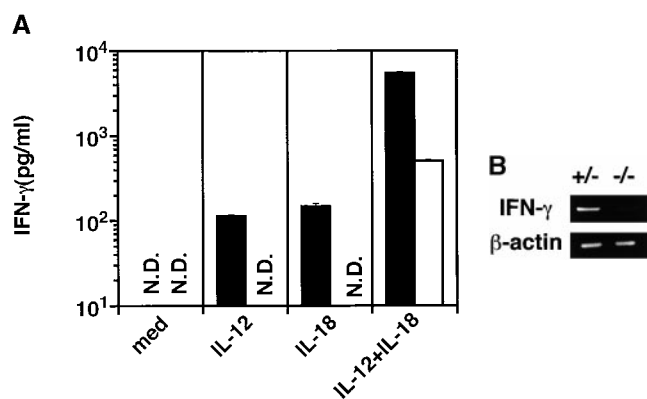
**Reduced Ability of  $C/EBP\gamma^{-/-}$  Chimeras Splenocytes to Produce  $IFN-\gamma$  in Response to  $IL-12$  and/or  $IL-18$ .** NK cells constitutively express both functional  $IL-12R$  and  $IL-18R$ , whereas naive T cells do not (21, 25, 26). Therefore, splenic  $IFN-\gamma$  production by stimulation with  $IL-12$  and  $IL-18$  is dependent on NK but not T cells. To evaluate the ability of NK cells from chimeric mice to produce  $IFN-\gamma$ , splenocytes were cultured with or without  $IL-12$  and/or  $IL-18$ . 24 h later, cell-free supernatants were harvested and assayed for  $IFN-\gamma$  production with ELISA. Under this condition,  $C/EBP\gamma^{-/-}$  chimeras splenocytes produced much lower amounts of  $IFN-\gamma$  than  $C/EBP\gamma^{+/+}$  chimeras splenocytes (Fig. 4 A).  $IL-12$  or  $IL-18$  can induce  $IFN-\gamma$  production at the transcriptional level in NK cells (27). Consistent with reduced  $IFN-\gamma$  production, induction of  $IFN-\gamma$  mRNA was markedly decreased in  $C/EBP\gamma^{-/-}$  chimeras splenocytes (Fig. 4 B). Taken together, these results suggest



**Figure 3.** NK cell population and cytotoxic activity of  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  chimeras. (A) Single-cell suspensions from the chimera splenocytes were stained with FITC-anti-CD3 and PE-anti-IL-2R $\beta$  Ab (top panels) or with FITC-anti-CD3 and biotin-DX5 followed by PE-streptavidin (bottom panels). The percentages of NK cell population are shown. (B) YAC-1 killing analysis of spleen cells.  $C/EBP\gamma^{+/+}$  and  $-/-$  chimera spleen cells were incubated without any cytokines for 4 h or with 2 ng/ml IL-12, 20 ng/ml IL-18, 2 ng/ml IL-12 plus 20 ng/ml IL-18, or 500 U/ml IL-2 for 24 h, and their cytotoxic activities against YAC-1 cells were determined. E/T ratios are shown on the x-axis. Six independent experiments were performed with similar results. The data indicate mean  $\pm$  SD of triplicate samples of one representative experiment.

that  $C/EBP\gamma$  is required for induction of IFN- $\gamma$  by IL-12 and/or IL-18 in NK cells.

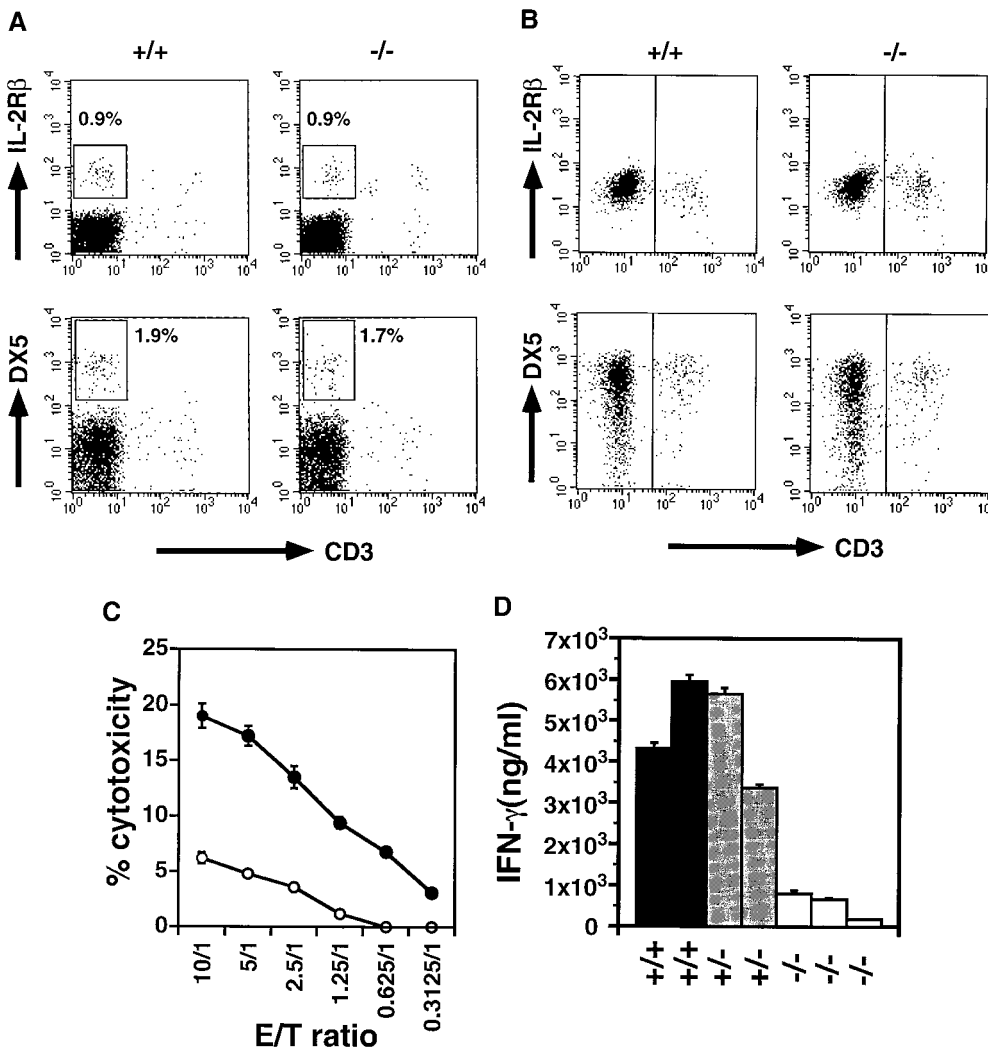
**IL-15-induced NK Cells from  $C/EBP\gamma^{-/-}$  Newborn Spleens Showed Impairment of Both Cytotoxic Activity and IFN- $\gamma$  Production.** NK cell population was analyzed in newborn



**Figure 4.** IFN- $\gamma$  production by chimera splenocytes in response to IL-12 and/or IL-18. (A) Spleen cells from  $C/EBP\gamma^{+/+}$  (filled columns) and  $C/EBP\gamma^{-/-}$  (open column) chimera splenocytes were cultured in the absence (med) or presence of 2 ng/ml IL-12 and/or 20 ng/ml IL-18 for 24 h. Amounts of IFN- $\gamma$  in the culture supernatants were measured by ELISA. Experiments were independently performed six times with similar results. The data indicate mean  $\pm$  SD of triplicate samples of one representative experiment. N.D., not detected. (B) RT-PCR analysis of  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  chimera splenocytes cultured with 2 ng/ml IL-12 and 20 ng/ml IL-18 for 24 h. Total RNAs were isolated and analyzed for IFN- $\gamma$  and  $\beta$ -actin expression by RT-PCR. Experiments were independently performed three times with similar results.

splenocytes. Both CD3<sup>+</sup>IL-2R $\beta$ <sup>+</sup> and CD3<sup>+</sup>DX5<sup>+</sup> cells were equivalently detected in  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  newborn splenocytes (Fig. 5 A). IL-15 can stimulate NK cell activity and proliferation and is essential for NK cell development (28, 29). Adult bone marrow cells can generate NK cells when cultured with IL-15 (28, 30). Wild-type newborn spleen cells could also give rise to CD3<sup>+</sup>IL-2R $\beta$ <sup>+</sup>DX5<sup>+</sup> cells in the presence of IL-15 (Fig. 5 B). In this culture condition, harvested cell numbers from control ( $C/EBP\gamma^{+/+}$ ,  $n = 5$ , and  $C/EBP\gamma^{+/-}$ ,  $n = 3$ ) and  $C/EBP\gamma^{-/-}$  ( $n = 7$ ) mouse spleen cells were  $5.7 \pm 4.1 \times 10^5$  and  $4.9 \pm 6.7 \times 10^5$  per well, respectively. Surface phenotype of cultured  $C/EBP\gamma^{-/-}$  cells was identical to that of wild-type cells (Fig. 5 B). However, NK cells generated from  $C/EBP\gamma^{-/-}$  spleens showed impaired cytotoxic activity against YAC-1 cells as compared with those from  $C/EBP\gamma^{+/+}$  spleens (Fig. 5 C). Furthermore,  $C/EBP\gamma^{-/-}$  NK cells produced lower amounts of IFN- $\gamma$  in response to IL-12 plus IL-18 than  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{+/-}$  NK cells (Fig. 5 D). Taken together, two major NK cell activities, cytotoxic activity and IFN- $\gamma$  production, were impaired in both  $C/EBP\gamma^{-/-}$  chimera splenocytes and  $C/EBP\gamma^{-/-}$  NK cells generated in the presence of IL-15 in vitro.

**IL-12 and IL-18 Signaling Analysis of  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  NK Cells.** To determine if impaired induction of IFN- $\gamma$  by IL-12 and IL-18 is caused by decreased expression of these receptors, we examined their mRNA expression in  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  NK cells. IL-12R $\beta$ 1, IL-12R $\beta$ 2, and IL-18R expression was equivalent in  $C/EBP\gamma^{+/+}$  and  $C/EBP\gamma^{-/-}$  NK cells (Fig. 6 A). IL-12 can



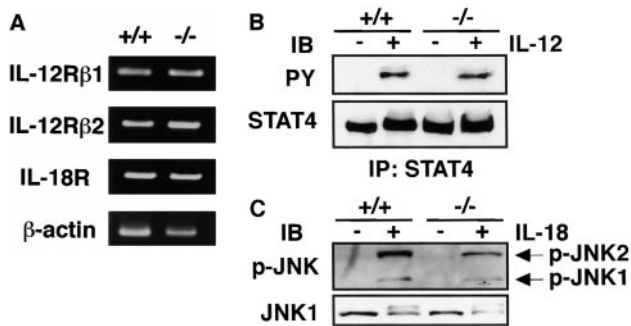
**Figure 5.** Characterization of IL-15-induced NK cells from newborn spleen cells. (A) Flow cytometric analysis of newborn spleen cells. Splenocytes were taken within 12–18 h after birth and analyzed for IL-2R $\beta$ , DX5, and CD3 expressions as described in the legend to Fig. 3 A. The percentages of NK cell population are shown. (B) Flow cytometric analysis of in vitro NK cells. Newborn spleen cells cultured with IL-15 for 10 d were analyzed using FACScalibur™ with CELL-Quest™ software. (C) YAC-1 killing analysis of in vitro NK cells. C/EBP $\gamma$ <sup>+/+</sup> (●) and C/EBP $\gamma$ <sup>-/-</sup> (○) NK cells were analyzed for their cytotoxic activities. The data indicate mean  $\pm$  SD of triplicate samples of one representative experiment. (D) IFN- $\gamma$  production by C/EBP $\gamma$ <sup>+/+</sup>, C/EBP $\gamma$ <sup>+/-</sup>, and C/EBP $\gamma$ <sup>-/-</sup> NK cells. IL-15-induced NK cells were harvested and cultured at 10<sup>5</sup> cells/well in 96-well plates for 24 h in the presence of 2 ng/ml IL-12 and 20 ng/ml IL-18. Amounts of IFN- $\gamma$  in the culture supernatants were measured by ELISA. The representative data from the same littermates are shown as mean  $\pm$  SD of triplicate samples. We analyzed four C/EBP $\gamma$ <sup>+/+</sup>, eight C/EBP $\gamma$ <sup>+/-</sup>, and five C/EBP $\gamma$ <sup>-/-</sup> mice for A, five C/EBP $\gamma$ <sup>+/+</sup>, three C/EBP $\gamma$ <sup>+/-</sup>, and seven C/EBP $\gamma$ <sup>-/-</sup> mice for B and D, and three C/EBP $\gamma$ <sup>+/+</sup>, one C/EBP $\gamma$ <sup>+/-</sup>, and four C/EBP $\gamma$ <sup>-/-</sup> mice for C and obtained similar results.

induce tyrosine phosphorylation and activation of STAT4 (31, 32). This pathway was not impaired in C/EBP $\gamma$ <sup>-/-</sup> NK cells (Fig. 6 B). IL-18 can cause phosphorylation and activation of JNK (33). Equivalent JNK phosphorylation was observed in C/EBP $\gamma$ <sup>+/+</sup> and C/EBP $\gamma$ <sup>-/-</sup> NK cells when stimulated by IL-18 (Fig. 6 C). Taken together, these results suggest that signaling pathways proximate to these cytokine receptors are intact in C/EBP $\gamma$ <sup>-/-</sup> NK cells.

In this study, we demonstrate that C/EBP $\gamma$ <sup>-/-</sup> NK cells have defects in IFN- $\gamma$  production and cytotoxicity. It has been shown that several regulatory elements such as the activator protein (AP)-1 or NF- $\kappa$ B sites are essential for IFN- $\gamma$  gene expression by IL-12 and/or IL-18 (34, 35). Although NF-IL6 is a candidate for heterodimerizing with C/EBP $\gamma$  (36), it seems unlikely that the heterodimer plays an essential role. First, no C/EBP sites have been shown to be important for IFN- $\gamma$  induction. Second, splenic IFN- $\gamma$  production in response to IL-12 and/or IL-18 is not impaired in NF-IL6<sup>-/-</sup> mice (our unpublished data). It is possible that C/EBP $\gamma$  plays a critical role in IFN- $\gamma$  gene regulation by dimerizing with AP-1 components. AP-1 components are

activated by IL-12 or IL-18 and essential for both IL-12- and IL-18-induced IFN- $\gamma$  promoter activation (34). Fos or Jun is shown to require C/EBP $\gamma$  in order to efficiently bind to the regulatory element in the IL-4 promoter (12). Although further studies are necessary, our results suggest that C/EBP $\gamma$  regulates IFN- $\gamma$  gene expression. At present, the possibility that C/EBP $\gamma$  is necessary for expression of other gene(s) critical for IFN- $\gamma$  gene induction cannot formally be excluded.

IFN- $\gamma$  does not seem to be involved in cytotoxic activity of NK cells, because NK activity is not remarkably impaired in IFN- $\gamma$ <sup>-/-</sup> mice (21). Therefore, impaired IFN- $\gamma$  production cannot account for decreased cytotoxic activity of C/EBP $\gamma$ <sup>-/-</sup> NK cells. Although CD18 is important for NK cells to recognize target cells (37), surface expression of CD18 was not decreased in C/EBP $\gamma$ <sup>-/-</sup> chimera splenocytes (data not shown). Furthermore, IL-12 and IL-18 could induce expression of a critical cytolytic mediator, perforin, in C/EBP $\gamma$ <sup>-/-</sup> chimera splenocytes (data not shown). These results indicate the presence of other target(s) of C/EBP $\gamma$  that are involved in the cytotoxicity of NK cells.



**Figure 6.** IL-12 and IL-18 signaling of IL-15-induced NK cells. (A) Total RNAs were purified from 10-d IL-15-cultured newborn spleen cells and analyzed for IL-12R $\beta$ 1, IL-12R $\beta$ 2, IL-18R, and  $\beta$ -actin expression by RT-PCR. (B) Cells were stimulated with or without IL-12 for 20 min, and the cell lysates were immunoprecipitated with anti-STAT4 Ab. The immunoprecipitates were separated on SDS-PAGE and blotted with antiphosphotyrosine Ab (top) or anti-STAT4 Ab (bottom). (C) Cells were or were not stimulated with IL-18 for 20 min. The cell lysates were blotted with antiphospho-JNK Ab (top) or anti-JNK1 Ab (bottom). Experiments were independently performed three times with similar results.

It is noteworthy that in spite of NK cell dysfunction, NK cell generation is intact in the absence of C/EBP $\gamma$ . This means that C/EBP $\gamma$  should play critical roles in a late maturation step rather than in an early developmental step. In contrast, mutant mice established so far manifested impaired NK cell generation. The IL-15 receptor consists of IL-15R $\alpha$ , IL-2R $\beta$ , and the common  $\gamma$  chain (38–42). Deficiency of one of these components caused reduction of NK cell num-

bers in vivo and impaired NK cell expansion in vitro (43–45). Janus kinase (JAK)-3 and STAT5 are essential for IL-15 signaling components. JAK-3 $^{-/-}$  or STAT5 $^{-/-}$  mice also showed impairment of NK cell generation (23, 46–49). Furthermore, IFN regulatory factor (IRF)-1 was found to be critical for expansion of NK cells (19). This can be explained by impaired IL-15 production in the bone marrow microenvironment (19). In these mutants, deficient NK cell generation is caused by disturbance of IL-15 signaling or decreased IL-15 production. Membrane lymphotoxin (LT) $\alpha$  also plays essential roles for NK cell development by acting independently of IL-15/IRF-1 or upstream of the IL-15/IRF-1 pathway (50). NK cell generation was severely impaired in LT $\alpha$  $^{-/-}$  mice. Furthermore, NK cells, which were generated in vitro with IL-15 from LT $\alpha$  $^{-/-}$  bone marrow cells, showed intact cytotoxic activity. These characteristics in LT $\alpha$  $^{-/-}$  mice are distinct from those in C/EBP $\gamma$  $^{-/-}$  mice. In addition, there are two more mutants with impaired NK cell development, the mechanism of which is not yet clear. One is deficiency of a winged helix-turn-helix transcriptional factor, Ets-1 (51). The other is deficiency of Id2, an inhibitor for transcription factors with helix-loop-helix domains (52).

At present, little is known about the molecular mechanisms that regulate NK cell functions or development. Our study clearly reveals that C/EBP $\gamma$  is critically involved in functional NK cell maturation. Identification of the target genes regulated by C/EBP $\gamma$  will clarify the molecular mechanism of NK cell functions.

We thank Ms. Akiko Maekawa, Ms. Nana Iwami, and Ms. Shizue Futatsugi for excellent technical assistance and Drs. Mitsuru Matsumoto, Yoshiko Murakami, Teruhito Yasui, and Hitoshi Kikutani for useful advice about transfer experiments. We are also grateful to Drs. Frederick W. Alt, Shigekazu Nagata, and Makoto Matsumoto for providing RAG2 $^{-/-}$  mice, the plasmid for C/EBP $\gamma$  genomic fragments, and PCR primers for  $\beta$ -actin, respectively.

This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan, Uehara Memorial Foundation, Novartis Foundation for the Promotion of Science, Kato Memorial Bioscience Foundation, and CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation.

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Submitted: 1 June 1999 Revised: 5 August 1999 Accepted: 21 September 1999

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