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IFN Regulatory Factor-2 Deficiency Revealed a Novel Checkpoint Critical for the Generation of Peripheral NK Cells

Shinsuke Taki,2* Shinsuke Nakajima,* Eri Ichikawa,* Takashi Saito,† and Shigeaki Hida*

NK cell development is far less understood compared with that of T and B cells despite the critical importance of NK cells in innate immunity. Mice lacking the transcription factor IFN regulatory factor-2 (IRF-2) are known to exhibit NK cell deficiency. However, the role of IRF-2 in NK cell development has remained unclear. In this study we found that NK cell deficiency in the periphery in IRF-2-deficient mice was due to selective loss of mature NK cells, but not to maturation arrest, and NK cells in these mice exhibited very immature surface phenotypes (CD11b^low^Dx5^low^) with highly compromised NK receptor expression. In contrast, IRF-2-deficient NK cells in bone marrow (BM) showed relatively mature phenotypes (CD11b^high^Dx5^high^) with less compromised NK receptor repertoire. Furthermore, BM NK cells in IRF-2-deficient mice were found to proliferate almost normally, but underwent accelerated apoptosis. These observations indicated that NK cell maturation could advance up to a late, but not the final, stage in the BM, whereas these cells were incapable of contributing to the peripheral NK cell pool due to premature death in the absence of IRF-2. In contrast, NK cell numbers and Ly49 expression were much more severely reduced in BM in IL-15-deficient mice than in IRF-2^−/−^ mice. The differential peripheral and central NK cell deficiencies in IRF-2^−/−^ mice thus revealed a novel late checkpoint for NK cell maturation, distinct from the early IL-15-dependent expansion stage. The Journal of Immunology, 2005, 174: 6005–6012.
IL-15 was essential for early expansion of NK cells. Functional relationships between IRF-2 and GATA-3, another transcription factor required for mature NK cell generation (16), will be discussed.

Materials and Methods

**Mice**

IRF-2−/− mice (17) backcrossed to C57BL/6 mice at least six times were described previously (18). Mice lacking β2-microglobulin (β2m−/− mice) and RAG-1 (RAG-1−/− mice) were purchased from The Jackson Laboratory and were provided by Dr. T. Nakayama (Chiba University, Chiba, Japan), respectively; they were backcrossed to C57BL/6 mice at least six times in addition and used to generate IRF-2−/− β2m−/− and IRF-2−/− RAG-1−/− double-mutant mice. IRF-2−/− H-2d mice were generated by intercrossing IRF-2−/− mice with B10.D2 mice (SLC). IL-15−/− mice (13) were purchased from Taconic Farms. As we observed no significant difference between IRF-2−/− and IRF-2+/+ mice, with either of these two genotypes were used as controls. All mice were maintained in the animal facility in Shinshu University under specific pathogen-free conditions and used at 8–12 wk of age according to the institutional guideline for animal experimentation.

**Abs and reagents**

Fluorochrome- and biotin-conjugated mAbs and streptavidins used in this study were purchased from BD Pharmingen, except for FITC-anti-Ly5.2, PE-anti-Ly49H (clone YLI-90), anti-c-Kit, and anti-TCRβ from eBioscience. Biotin-labeled Abs were developed with PE-Cy7- or PE-Texas Red-streptavidin (eBioscience). Anti-CD16/32 Ab (FcBlock; BD Pharmingen) was used to block Fc-mediated binding of mAbs.

**Flow cytometry**

BM cells were flushed from the femurs, PBMC were isolated by centrifugation over Percoll (Amerham Biosciences), and liver mononuclear cells were prepared as previously described (19). Cells were stained and analyzed on the Cytometers FC500 flow cytometer (Beckman Coulter). Dead cells were regularly gated out using propidium iodide, and cells within the lymphocyte gate defined by forward and side scatters were analyzed. Data analysis was conducted with RXP software (Beckman Coulter).

**Natural cytotoxicity and IFN-γ production**

Splenical and BM NK cells were enriched from control RAG-1−/− and IRF-2−/− RAG-1−/− double-mutant mice by depleting TCRβ+, CD19+, TER119+, and F-A++ cells using MACS (AutoMACS; Miltenyi Biotec), and natural cytotoxicity on YAC-1 cells was examined as previously described (20). Because the purity of NK cells was not always identical in the preparations from control RAG-1−/− and IRF-2−/− RAG-1−/− double-mutant mice due to the reduced numbers of NK cells in the latter, killer assays were conducted with the defined NK cells to target cell ratios. IFN-γ production by NK cells was examined on the flow cytometer by staining spleens or BM cells cultured for 16 h in the presence of recombinant human IL-2 (1000 U/ml; provided by Ajinomoto) and murine recombinant IL-12 (5 ng/ml; R&D Systems). Surface-stained cells for NK1.1, CD3, and CD11b were subjected to cytoplasmic staining for IFN-γ using a Cytofix/ Cytoperm Plus kit (BD Pharmingen) according to the instructions supplied by the manufacturer.

**Radiation BM chimeras**

Radiation BM chimeras were established by reconstituting irradiated B6-Ly5.1 mice (purchased from Sankyo) with RBC-depleted BM cells prepared from IRF-2−/− or control mice bearing Ly5.2 marker as previously described (18) and were analyzed 8–10 wk later. In all chimeras, the percentages of donor-derived Ly5.1+ cells within splenic and BM NK cells exceeded 95%.

**In vivo proliferation and apoptosis**

In vivo proliferation of NK cells was examined as previously described (6). Briefly, BrdU (Wako) solution in PBS was injected i.p. (3 mg/head), and 3 h later, BM cells were isolated and stained using a BrdU flow kit (BD Pharmingen) according to the instructions provided by the supplier. To detect cells undergoing apoptosis, NK1.1+/CD3− cells were electrically gated and examined with PE-labeled annexin V using an Apoptosis Detection Kit (BD Pharmingen). Already dead cells positive for both 7-aminoactinomycin and annexin V were excluded from the analyses.

Allogenic BM graft rejection

Lethally irradiated (9.0 Gy) IRF-2−/− or control mice were injected i.v. with 3 × 10^6 RBC-depleted BM cells prepared from BALB/c mice. Eight days later, spleens were isolated and fixed in Bouin’s solution.

**Results**

Residual NK cells in the periphery in IRF-2−/− mice lacked phenotypically mature cells

As previously reported (12), IRF-2−/− mice exhibited a reduction of NK cells defined as NK1.1+ CD3− cells in spleen and liver (Fig. 1, A and B). We examined the developmental stages of the residual NK cells in the periphery in IRF-2−/− mice according to the developmental framework proposed by Yokoyama et al. (2, 6), in which developmental intermediates of NK cells up-regulated the expression of Dx5 first and then that of CD11b, followed by acquisition of CD43. As shown in Fig. 1, A and B, splenic and hepatic NK cell populations in control animals contained small numbers of immature NK cells (CD43− CD11blow) together with the prevailing numbers of fully mature NK cells (CD43+ CD11bhighDx5high), an observation consistent with the report by Kim et al. (6). In contrast, the number of not only CD11bhigh Dx5high, but also that of CD11bhighDx5high, NK cells was greatly reduced, and virtually no NK cells were positive for CD43 in spleen and liver in IRF-2−/− mice (Fig. 1, A and B). Notably, the absolute numbers of CD11b+immature NK cells in individual mice exceeded 95%.

**FIGURE 1.** Differential impairment of immature and mature NK cells in the periphery in IRF-2−/− mice. Surface phenotypes of NK cells in spleen (A) and liver (B) are shown. The percentages of NK1.1+ CD3− in total lymphocytes are shown in the top row. Plots in the second and bottom rows show the profiles of NK cells gated in the top row. The numbers represent the percentages of cells in each quadrant. Representative data for >10 pairs of mice are presented. C, The absolute numbers of the CD43− CD11b+ (immature) and CD43+ CD11b+ (mature) NK cell subpopulations as well as total NK cells in each spleen were calculated for control (○) and IRF-2−/− (●) mice. Each symbol represents the value for an individual mouse. Horizontal bars are the means.
spleens in IRF-2−/− mice were not dramatically altered, whereas those of CD11bhigh mature NK cells were 100-fold reduced, on the average, compared with those in control animals (Fig. 1C). It was of note that more than half the splenic and hepatic NK cells in IRF-2−/− mice were Dx5low in contrast to control NK cells, >80% of which were Dx5high. These observations indicated that IRF-2 deficiency affected not only phenotypically mature NK cell production, but also immature NK cells qualitatively.

Normal cytotoxicity and inefficient IFN-γ production of phenotypically immature NK cells in IRF-2−/− mice

Phenotypically immature NK cells enriched from the spleen (Fig. 2A) or BM (Fig. 2B) of IRF-2−/−RAG-1−/− double-mutant mice were similarly cytotoxic to NK-sensitive YAC-1 cells as those from RAG-1−/− mice. In contrast, combined stimulation with IL-2 and IL-12 in vitro induced IFN-γ-producing NK cells less efficiently from splenocytes derived from IRF-2−/− mice (16.2%) than from control splenocytes (26.0%; Fig. 2C). Although the majority of IFN-γ-producing NK cells in the control cultures were CD11bhigh, considerable fractions of CD11blow NK cells also producing IFN-γ, in agreement with a previous report (6). However, IFN-γ producers were less prominent in the CD11bhigh fraction in IRF-2−/− mice than in the corresponding fraction in control mice (13.5/89.7 vs 4.7/21.0; Fig. 2C). A similar reduction in IFN-γ production was observed in CD11blow NK cells in the BM of IRF-2−/− mice (Fig. 2D). Furthermore, rejection of allogenic BM grafts in IRF-2−/− mice was highly inefficient compared with that in controls, as revealed by the elevated numbers of CFUs in the spleen in the former (Fig. 2E). This observation was consistent with a previous description that rejection of the MHC-negative tumor RMA-S was strongly impaired in IRF-2−/− mice (12).

Thus, the lack of IRF-2 affected the physiological functions of peripheral NK cells in vivo despite the normal natural cytotoxicity.

NK cells in BM exhibited relatively mature phenotypes compared with splenic and hepatic NK cells in IRF-2−/− mice

We observed that CD51 and c-Kit were not yet down-regulated on the majority of NK cells in the BM in IRF-2−/− mice (Fig. 3A), and these cells lacked the fully mature CD43+CD11bhigh cells, as found in our preliminary analyses (18) (Fig. 3A), indicative of the immature nature of these NK cells (2, 6). Quantitatively, the numbers of CD11blow immature NK cells were reduced in the BM of IRF-2−/− mice to only half the number in control mice (Fig. 3B; 5.9 vs 10.5 × 104 cells/femur), whereas those of CD43+CD11bhigh fully matured NK cells were 50-fold reduced in the absence of IRF-2 (Fig. 3B; 0.92 vs 43.8 × 104 cells/femur), indicating that generation from the committed progenitors and expansion of immature NK cells were not severely impaired, but further maturation was drastically impaired in IRF-2−/− mice. On the average, CD51+ and c-Kit+ cells constituted 62.6% and 71.3% of the CD11blow NK cells in BM NK cells in IRF-2−/− mice (n = 3), respectively, whereas CD11bhigh NK cells in control mice were only 30.9 and 35.5% positive for CD51 and c-Kit, respectively (n = 3). Thus, the absolute numbers of CD51+CD11bhigh and c-Kit+CD11bhigh NK cells were calculated to be 3.7 and 4.2 × 104 cells/femur, respectively, for IRF-2−/− mice and 3.2 and 3.7 × 104 cells/femur, respectively, for control mice. These values were not dramatically different between IRF-2−/− and control mice, suggesting that IRF-2 deficiency mainly affected the transition from the CD51/c-Kit+ to the CD51/c-Kit− stage within CD11blow NK cells. This was a situation contrasting with that in IL-15−/− mice, in which the total numbers of NK cells in the BM were 40- and 10-fold more severely reduced than in control and IRF-2−/− mice, respectively (Fig. 3B). Because practically all NK cells in the BM in IL-15−/− mice were CD11blow (S. Taki, unpublished observation), these results indicated that IL-15 was required for early expansion, whereas IRF-2 acted later for the maturation of NK cells. Importantly, the majority of CD11blow immature NK cells in the

FIGURE 2. Normal cytotoxicity, but reduced production, of IFN-γ by splenic NK cells in IRF-2−/− mice. A. NK cells enriched from the spleens of control RAG-1−/− (○; NK cells, 53.1%) or IRF-2−/− RAG-1−/− double-mutant (●; NK cells, 53.3%) mice were tested for their cytotoxicity on YAC-1 cells. The NK cell/target ratio was adjusted according to the percentage of NK cells within the effector cell population. Each symbol represents the mean ± SD of duplicated cultures. The data shown are representative of more than 12 independent experiments. B. BM NK cells were enriched from RAG-1−/− mice (○; NK cells, 53.5%) or IRF-2−/− RAG-1−/− double-mutant mice (●; NK cells, 40.5%) and examined for natural cytotoxicity as described in A. The data shown are representative of four totally independent experiments with similar results. C. BM NK cells were stimulated with IL-2 alone (−) or with IL-2 and IL-12 (IL-12) and stained for cytoplasmic IFN-γ. Representative data are shown for NK1.1+CD3− cells of three independent experiments involving five mice of each genotype in total. The numbers represent the percentage of cells in each quadrant. D. BM cells were examined for IFN-γ production as described in C. The data presented are representative of two independent experiments. E. Spleens isolated from lethally irradiated mice 8 days after injection of allogenic BM cells are shown. Note that several discrete colonies were seen in the spleens from control mice, whereas it was difficult to identify single colonies in the spleens from IRF-2−/− mice, because too many colonies were present, and they were fused to each other. The data shown are representative of more than four recipients of each genotype.
BM in IRF-2−/− mice were Dx5high (Fig. 3A) in contrast with those in the spleen and liver that stayed at the Dx5low stage (Fig. 1, A and B). Because Dx5 were shown to be one of the earliest markers acquired during NK cell development (6, 8), these results indicated unexpectedly that NK cell development advanced up to the CD11blowDx5high stage in the BM, but these cells did not efficiently contribute to the peripheral NK cell pool.

Differential abnormalities between splenic and BM NK cells in IRF-2−/− mice were due to the hemopoietic cell-intrinsic IRF-2 deficiency

Similar differences in surface phenotypes between BM and splenic NK cells were observed in radiation BM chimeras established by reconstituting B6-Ly5.1 mice with BM cells from control and IRF-2−/− mice (control and IRF-2−/− chimeras, respectively; Fig. 4). Thus, Dx5low NK cells were dominant in the spleen in IRF-2−/− chimeras (Fig. 4A), whereas Dx5high NK cells were prevailing in the BM (Fig. 4B). In addition, the percentages of CD43+ and CD43−CD11bhigh NK cells derived from the donors were much lower in the spleen in IRF-2−/− chimeras than in control chimeras (Fig. 4A). Another series of chimeras generated with the converse host/donor combination in two independent experiments.

Differential distortion of the NKR repertoire in spleen and BM in IRF-2−/− mice

We further characterized NK cells in IRF-2−/− mice by examining the expression of NKRs that were critical for physiological functions of NK cells (9, 21). As shown in Fig. 5A, Ly49 receptor expression by splenic NK cells was much less frequent in IRF-2−/− mice than in control mice. On the contrary, NK cells expressing NKG2A/C/E were more frequent in IRF-2−/− than in control mice. Another NKG2 molecule, NKG2D, was expressed by all NK cells regardless of IRF-2 deficiency (S. Nakajima, unpublished observation). Notably, in contrast, the frequencies of Ly49 receptors, except for Ly49I, on IRF-2-deficient NK cells in the BM were significantly higher than those in control mice, and Ly49l- and NKG2A/C/E-expressing NK cells were less frequent in IRF-2−/− mice than in control mice (Fig. 5A). Because the majority of NK cells in IRF-2−/− mice were CD11blow, we also compared Ly49 expression between control and IRF-2-deficient CD11bhigh NK cells (Fig. 5B). Even in these cases, the frequencies of Ly49− NK cells in the spleen were affected in a manner similar to that in whole NK cells, albeit with a reduced severity, probably reflecting the immature nature of CD11bhigh splenic NK cells in IRF-2−/− mice, as indicated by the Dx5low phenotype (Fig. 1). These results indicated that both the lack of CD11bhigh NK cells and the immaturity of CD11bhigh NK cells contributed to the reduced Ly49+ NK cell frequencies in the spleen in IRF-2−/− mice. As previously shown, CD11bhigh immature NK cells expressed Ly49 receptors and NKG2A/C/E at lower and higher frequencies, respectively, compared with CD11bhigh
Normal selection of inhibitory Ly49 receptor repertoire in BM in IRF-2−/− mice

The inefficient contribution of BM Ly49+ NK cells to the peripheral NK cell population in IRF-2−/− mice (Fig. 5A) raised the possibility that MHC class I-mediated self tolerance (7, 22) might select negatively Ly49+ cells. As summarized in Table I, the presence of H-2d molecules, ligands for Ly49A and G2 receptors (23), significantly reduced the frequencies of NK cells expressing these Ly49 receptors in the BM regardless of the presence of IRF-2. Consistent with the hypothesis that class I MHC-mediated self tolerance acted to reduce the frequencies of NK cells expressing multiple Ly49 receptors (24–26), the prevalence of Ly49A+G2+ double-positive (DP) cells was lower in mice expressing H-2d molecules than in β2m−/− mice regardless of whether IRF-2 was present (Table I). This decrease was not due merely to the reduced frequencies of each Ly49 receptor, because the deviation of the observed Ly49A+G2+ DP cell frequencies from the values expected assuming random distribution of each Ly49 receptor was lower in mice with the H-2d background than in β2m−/− mice (Table I). Thus, even in the absence of IRF-2, class I-dependent counterselection against the developing Ly49A+G2+ DP cells occurred normally in the BM.

In contrast, the frequencies of Ly49I+ NK cells were only marginally affected by H-2d molecules in both control and IRF-2−/− mice (Table I, compare H-2d and β2m−/− mice), although Ly49I was known to bind H-2d, albeit weakly (7, 23). These observations suggested that the selection against Ly49I+ NK cells had not been established yet in the BM, possibly because of the relatively late generation of Ly49I+ NK cells during NK cell development, in line with the models in which various Ly49 receptors were expressed sequentially but not simultaneously (4, 5). This idea was consistent with the observation that Ly49I+ NK cells in the BM were, in contrast to Ly49A+ or G2+ NK cells, less frequent in IRF-2−/− mice that lacked mature NK cells than in control mice regardless of the MHC genotype (Fig. 5A and Table I).

Activated phenotypes and accelerated apoptosis of IRF-2-deficient NK cells in vivo

We further characterized NK cells in BM in IRF-2−/− mice. It was found that BM NK cells in IRF-2−/− mice were apparently larger than those in control mice, as judged from the forward scatter profile (Fig. 6A). In addition, the frequencies of CD11b+ imm mature NK cells in the BM expressing CD69 were elevated in IRF-2−/− mice compared with those in control mice (Fig. 6B). These observations indicated that BM immature NK cells in IRF-2−/− mice were hyperactivated. However, this hyperactivated state of immature NK cells was not associated with accelerated proliferation, because the frequencies of NK cells acutely incorporating

Table I. Influence of MHC on the frequencies of NK cells bearing inhibitory Ly49 receptors in the BM in IRF-2−/− mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>% in NK Cellsa</th>
<th>% Ly49A+/ G2+ in NK Cellsa</th>
<th>% Ly49A+ / G2+ in NK Cellsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-H-2√</td>
<td>18.3 ± 1.7√</td>
<td>50.0 ± 4.9√</td>
<td>33.6 ± 2.9√</td>
</tr>
<tr>
<td>Control-β2m−/−</td>
<td>26.6 ± 2.6</td>
<td>67.0 ± 3.5</td>
<td>39.5 ± 4.2</td>
</tr>
<tr>
<td>IRF-2−/− H-2√</td>
<td>28.8 ± 3.2√</td>
<td>58.9 ± 4.1√</td>
<td>15.3 ± 3.1√</td>
</tr>
<tr>
<td>IRF-2−/− β2m−/−</td>
<td>33.2 ± 4.7</td>
<td>63.7 ± 0.9</td>
<td>17.2 ± 1.8√</td>
</tr>
</tbody>
</table>

a The numbers represent the mean ± SD of three mice for each genotype.

b Observed value/expected value.

c p < 0.05 compared with β2m−/− mice.

d p < 0.05 compared with the expected values calculated as the multiplied frequencies of the individual receptors.

e p < 0.05 compared with control mice with the same H-2 background.
The data shown are representative of three animals of each genotype. Capoptosis, as revealed by higher percentages of NK cells stained the loss of developing NK cells rather than developmental block-and CD69 expression (\( C \)) are shown for NK1.1 activated and undergoing accelerated apoptosis. Forward scatters (FSC; \( A \)) and CD69 expression (\( B \)) are shown for NK1.1\(^{\text{CD3}}\) BM cells prepared from the indicated mice. The data are representative of four animals of each genotype. C, BM cells were prepared 3 h after injection of BrdU and analyzed for BrdU incorporation. The numbers represent the percentages of BrdU positive cells within NK1.1\(^{\text{CD3}}\) cells. D, Cumulative data for BrdU-positive NK1.1\(^{\text{CD3}}\) BM cells from control (\( C \)) and IRF-2\(^{-/-}\) (\( B \)) mice as determined in C. Each symbol represents an individual mouse. E, The histograms show the profiles for annexin V binding of NK1.1\(^{\text{CD3}}\) mice in IRF-2, B, and D. In IRF-2\(^{-/-}\) mice, these NK cells were much more severely reduced in spleen as well as BM cells. The lower frequency of NK cells in IRF-2\(^{-/-}\) mice implied the loss of developing NK cells rather than developmental blockade in the absence of IRF-2, an idea supported by the accelerated apoptosis, as revealed by higher percentages of NK cells stained with annexin V (Fig. 6E).

**Discussion**

Although IRF-2 has long been known to be required for efficient NK cell development (12), the mechanism for IRF-2 function has remained unclear. In this study we observed, through quantitative examinations, that the absolute numbers of CD11b\(^{\text{high}}\) mature NK cells were much more severely reduced in spleen as well as BM than were those of CD11b\(^{\text{low}}\) immature NK cells (Figs. 1C and 3B). Because the percentages of proliferating NK cells in the BM in IRF-2\(^{-/-}\) mice were similar to those in control mice (Fig. 6, C and D), simple developmental arrest would have resulted in the accumulation of immature NK cells in the BM, as previously seen in other NK cell-deficient mice (16, 27). Hence, the severe and selective reduction of mature NK cells in IRF-2\(^{-/-}\) mice implied the loss of developing NK cells rather than developmental blockade in the absence of IRF-2, an idea supported by the accelerated apoptosis, as revealed by higher percentages of NK cells stained with annexin V (Fig. 6E).

Remarkably, NK cells in BM were more mature than those in spleen and liver in IRF-2\(^{-/-}\) mice, because the latter expressed less Dx5 than the former (Fig. 1, A and B, and Fig. 3A). The severer distortion of the NKR repertoire in spleen than in BM in IRF-2\(^{-/-}\) mice (Fig. 5A) supported this view. We propose a model for the role of IRF-2 in the generation of peripheral NK cells (Fig. 7) in which IRF-2 is dispensable for generating relatively mature CD11b\(^{\text{low}}\)Dx5\(^{\text{high}}\) NK cells that are expressing Ly49 receptors, whereas these cells cannot be efficiently exported to the periphery, because their premature apoptosis within the BM was accelerated in the absence of IRF-2 through a mechanism as yet to be established (see below). Selection of the Ly49 repertoire mediated by non-MHC and MHC factors seemed to proceed normally even in the BM in IRF-2\(^{-/-}\), at least for Ly49A and G2 (Table I), suggesting that the paucity of NK cells bearing Ly49 receptors (Fig. 5A) was not due to the aberrant NKR selection. Lowin-Kropf and Held (28) previously reported, in contrast, that forced over-expression of Ly49A had a positive impact on NK cell development. However, BM NK cells with Ly49A\(^{+}\) and G2\(^{+}\) cells of slightly elevated frequencies in IRF-2\(^{-/-}\) mice (Fig. 5A) appeared to have no developmental advantage. Thus, the influence of IRF-2 deficiency was dominant over the positive effects, if any, of Ly49 expression.

It was notable that NK cells bearing Ly49G2, A, and D in BM in IRF-2\(^{-/-}\) mice were even more frequent than those in control mice (Fig. 5A). These Ly49 receptors were known to be expressed at the CD11b\(^{\text{high}}\) stage (6). In fact, the frequencies of NK cells bearing these Ly49 receptors were only marginally elevated as NK cells up-regulated CD11b expression in control mice (Fig. 5, compare A and B). Because the majority of CD11b\(^{\text{low}}\) NK cells in the BM in IRF-2\(^{-/-}\) mice bore the immature NK cell markers CD51\(^{+}\) and c-Kit\(^{+}\) (Fig. 3A), the higher frequencies of Ly49G2\(^{+}\), A\(^{+}\), and D\(^{+}\) NK cells suggest that the expression of these Ly49 receptors peaked at very immature stages and was reduced as NK cells lost CD51 and c-Kit expression. Another interesting finding was that, in contrast to the other Ly49 receptors, Ly49I expression was infrequent in both BM and spleen in IRF-2\(^{-/-}\) mice compared with that in control mice regardless of the MHC background (Fig. 5, A and B, and Table I). A possible explanation for this observation would be that Ly49I expression occurred later during NK cell development than that of the other Ly49 receptors, seemingly during migration from BM to spleen (compare the data for spleen and BM in control mice in Fig. 5A). Transcriptional regulation of Ly49 receptors has not been well understood. It was previously shown.

![FIGURE 6. Immature NK cells in BM in IRF-2\(^{-/-}\) mice were hyper-activated and undergoing accelerated apoptosis. Forward scatters (FSC; A) and CD69 expression (B) are shown for NK1.1\(^{\text{CD3}}\) BM cells prepared from the indicated mice. The data are representative of four animals of each genotype. C, BM cells were prepared 3 h after injection of BrdU and analyzed for BrdU incorporation. The numbers represent the percentages of BrdU positive cells within NK1.1\(^{\text{CD3}}\) cells. D, Cumulative data for BrdU-positive NK1.1\(^{\text{CD3}}\) BM cells from control (C) and IRF-2\(^{-/-}\) (B) mice as determined in C. Each symbol represents an individual mouse. E, The histograms show the profiles for annexin V binding of NK1.1\(^{\text{CD3}}\) BM cells, and the numbers represent the percentages of annexin V\(^{+}\) cells. The data shown are representative of three animals of each genotype.](http://www.jimmunol.org/)

![FIGURE 7. A model for the NK cell deficiency in IRF-2\(^{-/-}\) mice. IRF-2 normally keeps CD11b\(^{\text{low}}\)Dx5\(^{\text{high}}\) cells expressing Ly49 receptors (B) alive, thereby allowing their exit to the periphery as well as further maturation into those expressing CD11b\(^{\text{high}}\) (C). In IRF-2\(^{-/-}\) mice, CD11b\(^{\text{low}}\)Dx5\(^{\text{high}}\) NK cells (B) are generated almost normally, but undergo premature cell death, and only very immature CD11b\(^{\text{low}}\)Dx5\(^{\text{low}}\) NK cells infrequently expressing Ly49 receptors (A) can reach the periphery. In contrast, IL-15 is involved in the expansion of immature NK cells at an earlier stage(s). GATA-3 may function in the transition from stage B to C (not shown). The circles with broken lines represent cells missing in IRF-2\(^{-/-}\) mice.](http://www.jimmunol.org/)
that Ly49A expression was under the selective control of the transcription factor TCF-1 (29). In this regard, uniquely impaired Ly49 expression in BM NK cells in IRF-2−/− mice might alternatively suggest that IRF-2 was a transcription factor selectively required for Ly49I, but not for the other Ly49, expression.

Despite the distinct phenotypic immaturities, NK cells in spleen as well as BM in IRF-2−/− mice were normally cytotoxic to YAC-1 cells (Fig. 2, A and B). This rather unexpected observation was, however, not peculiar to IRF-2−/− mice. It was also observed previously that natural cytotoxicity was unaffected or was only slightly reduced despite the impaired Ly49 expression and/or maturation defects in NK cells in mutant mice lacking PU.1, Id2, T-bet, or GATA-3 (16, 27, 30, 31). Furthermore, perforin mRNA was detected even in very primitive Dx5low NK cells in BM (8), and very immature NK cells in IL-15−/− mice have recently been shown to be cytotoxic, albeit at a reduced level (32). These observations by us and others suggested that acquisition of natural cytotoxicity was completed at early stages (Dx5low) of development, in contrast with IFN-γ production, which continued to be elevated at the transition from the CD11blow to the CD11bhigh stage (Fig. 2, C and D), and hence was not sufficient to define fully mature NK cells. An interesting possibility in this regard is that the severely impaired rejection of allogenic BM grafts (Fig. 2E) and the strong defect in MHC-negative tumor rejection in vivo (12) in IRF-2−/− mice might reflect defects in the maturation-associated expression and/or down-regulation of functional molecules in addition to the reduction of NK cell numbers (Fig. 1C) and IFN-γ production (Fig. 2C). This idea, however, is merely a speculation at this time, and it is impossible to exclude an alternative possibility that cells other than NK cells, which might contribute to allogenic BM rejection, were also affected in IRF-2−/− mice.

NK cells in IL-15−/− mice were defective in expressing Ly49A, G2 and I not only in the spleen as recently shown by Kawamura et al. (15) but also in the BM as observed in this study (Fig. 5C). Such a coordinate impairment of Ly49 expression between spleen and BM NK cells contrasted sharply to the differential impairment in Ly49 expression observed between BM and splenic NK cells in IRF-2−/− mice (Fig. 5, A and B). Thus, defective NK cell maturation, instead of the loss of Ly49+ NK cells like that in IRF-2−/− mice, seemed to be responsible for the distorted Ly49 repertoire of splenic NK cells in IL-15−/− mice (15). In addition to the Ly49-inducing activity, IL-15 was obviously critical also for early expansion of NK cells in the BM (Fig. 3B). Because the majority of proliferating NK cells in vivo were CD43+ Dx5+CD11bhigh (6) and the percentages of cells of this phenotype were higher in BM in IRF-2−/− mice than in control mice by a factor of ~1.5 (Fig. 3A), the unaltered percentages of proliferating NK cells (Fig. 6, C and D) indicated that NK cell proliferation was slightly less efficient in the absence of IRF-2. This partial impairment of proliferation might be due to the accelerated apoptosis (Fig. 6E) and could contribute in part to the severe loss of mature NK cells in the periphery. In contrast, we observed that the numbers of NK cells in the BM in IRF-2−/− mice were 10-fold more than those in IL-15−/− mice (Fig. 3B). We thus envisage that IL-15-mediated expansion takes place before IRF-2 acts (see Fig. 7) in a manner independent of IRF-2, and NK cell deficiency in IRF-2−/− mice is not due to the impaired IL-15 responses of immature NK cells, contrary to a previous hypothesis (12). The inefficient NK cell expansion from IRF-2-deficient BM cells in IL-15-supplemented cultures observed in the previous report (12) might be accounted for by accelerated apoptosis (Fig. 6E) and/or the low numbers of NK cells within BM cell preparations (Fig. 3B).

With respect to the regulation of cell survival, IRF-2 was previously shown to have an oncogenic potential as it transformed NIH-3T3 cells in vitro upon overexpression probably by repressing the proapoptotic actions of IRF-1 (33), another IRF family transcription factor shown to be required for NK cell development (34, 35). However, because IRF-1 acted in the BM microenvironment in a stem cell extrinsic manner during NK cell development (35), repression of IRF-1 actions by IRF-2 that was functioning within hematopoietic cells (12) (Fig. 4) appeared to be untenable in this case. IRF-2 was also implicated in the transcriptional activation of the cell cycle-regulated histone H4 gene, thereby potentially participating in cell cycle control (36). Thus, IRF-2 deficiency might result in cell cycle arrest, leading to the acceleration of apoptosis of immature NK cells independently of IRF-1. In relation to the antiapoptotic function of IRF-2 in developing NK cells, a recent report showed massive apoptosis of hepatic Kupffer cells occurring in IRF-2−/− mice treated with LPS in vivo (37).

It was of a great interest that the phenotypes of NK cells in IRF-2−/− mice closely resembled those in chimeric mice generated with GATA-3-deficient hemopoietic stem cells (16). Thus, in these chimeras, fully mature CD43+CD11bhigh NK cells were greatly reduced, and IFN-γ production by splenic NK cells was substantially lowered, whereas these cells exhibited virtually unaltered natural cytotoxicity. Moreover, NK cells expressing Ly49C/I and D were infrequent in these chimeras compared with those in controls. These strikingly common, albeit not completely overlapping, phenotypes suggested that GATA-3 and IRF-2 acted at similar stages of NK cell maturation. Nevertheless, the mechanisms of action of GATA-3 and IRF-2 did not seem to be identical, because the presence of Dx5high NK cells in spleen and the accumulation of phenotypically immature NK cells in BM were observed in GATA-3−/−/− chimeras (16), but not in IRF-2−/− mice (Figs. 1A and 3B). These dissimilar phenotypes suggested that GATA-3 was, unlike IRF-2, critical for the maturation, but not for the survival, of CD11bhighDx5high immature NK cells. Furthermore, because we observed unaltered expression of GATA-3 mRNA in purified IRF-2-deficient NK cells (S. Nakajima, unpublished observation), it seemed unlikely that GATA-3 was a direct target of IRF-2. We speculate that IRF-2 keeps developing NK cells surviving for a certain period of time by repressing apoptosis, thereby allowing GATA-3 to induce maturation. Although additional studies are certainly required for directly testing this hypothesis, IRF-2 deficiency pointed to a previously unrecognized checkpoint critical for establishment of the normal peripheral NK cell population. The target gene(s) of IRF-2 relevant to NK cell maturation is currently unknown, as are those of many other transcription factors required for efficient NK cell generation (16, 30, 31, 38, 39). We believe that identification of the target genes for IRF-2 relevant in NK cell maturation should contribute to unveiling the complicated gene regulatory network underlying NK cell maturation.

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Disclosures

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References


