The Transcription Factor Interferon Regulatory Factor-1 Is Essential for Natural Killer Cell Function In Vivo

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Summary

The activation of natural killer (NK) cells, cytotoxic lymphocytes capable of major histocompatibility complex (MHC)-unrestricted killing and early antiviral defense, is temporally related to the increased interferon (IFN)-α/β production that is seen in the viral infection of mice. Type I IFN (IFN-α/β) are expressed in many cell types early after primary viral infection and have been shown to mediate resistance against a variety of viruses. In this study, the role of the transcriptional activator IFN regulatory factor-1 (IRF-1) in murine NK cell activity was assessed. IRF-1−/− deficient mice displayed a normal frequency of NK marker-positive cells, but exhibited greatly reduced NK cell-mediated cytotoxicity after both virus infection and stimulation with the IFN inducer polyinosinic:polycytidilic acid in vivo. In vitro, cytolytic activity in IRF-1−/− NK cells remained defective after stimulation with IFN-β, IL-2, and IL-12. IRF-1−/− deficient mice were unable to eliminate syngeneic MHC class I-negative tumor cells in vivo, and had a reduced ability to reject parental semi-allogeneic donor cells from the circulation. Thus, IRF-1 is essential for the induction of NK cell-mediated cytotoxicity and for the in vivo effector functions that are mediated by this activity.

NK cells are cytotoxic lymphocytes that are capable of lysing a variety of target cells, including tumor cells, virus-infected cells, and cells infected with intracellular bacteria. NK cell-mediated killing does not require previous sensitisation and is MHC unrestricted (1), and as such, forms a first line of defense against microbial pathogens or tumor growth. In the case of primary viral infection, NK cell responses are induced early and are temporally distinct from those of T lymphocytes. The early response to viral infection is associated with increases in both systemic and local type I IFN (IFN-α/β) production, peaking at 3–5 d after infection, and closely mirroring the time course of NK cell activation, blastogenesis, and the augmented cytolytic activity that is seen in viral infection (2).

IFN regulatory factor-1 (IRF-1), an IFN-inducible transcriptional activator, binds regions within the promoters of type I IFNs and several IFN-inducible genes, and has been implicated as a mediator of the IFN-induced antiviral state (3, 4). IRF-1 is constitutively expressed in many cell types, and its expression can be strongly enhanced by type I and II IFNs, viral infection, double-stranded RNA, and cytokines such as IL-6 (4, 5). The functional deletion of IRF-1 does not, however, fully ablate the expression of IFN and IFN-inducible genes. We have previously demonstrated in vitro induction of type I IFN genes by polyinosinic:polycytidylid acid (pI:pC) and Newcastle disease virus to be IRF-1 dependent and independent, respectively (6). In addition, IRF-1−/− dependent antiviral effects of IFNs and the course of viral infection in IRF-1−/− deficient mice have been shown to be dependent on the type of virus investigated (7), suggesting IRF-1−/− dependent and -independent pathways in the induction of antiviral states.

To clarify the role of IRF-1 in NK cell function, we have examined the cytolytic activity of NK cells from IRF-1−/− mice in response to lymphocytic choriomeningitis virus (LCMV) and pI:pC in vivo, and to IFN-β, IL-2, and IL-12 in vitro. Killing of NK-sensitive targets in vitro by spleen NK cells from IRF-1−/− deficient mice was found to be markedly reduced both after in vivo and in vitro activation of NK cells. A defect was also evident in the clearance of parental donor cells from the circulation of IRF-1−/− deficient hosts and in the elimination of a syngeneic MHC class I-deficient tumor in vivo, both processes known to be mediated by NK cells. Our results clearly indicate that IRF-1 plays a crucial role in the induction of NK cell-mediated cytotoxic and effector functions in vivo.

Materials and Methods

Mice. C57BL/6 mice deficient in IRF-1 (6) were genotyped by genomic Southern blotting of tail DNA as described previ-
phocytes were gated on the basis of forward and side scatter char-
drazide. Cells were washed with PBS and analyzed by flow
acteristics, and 10,000 gated events were analyzed.
ated), anti-CD25 (7D4, baotinylated), and anti-CD11a (M17/4,
A/J F1, IRF-1 +/-, or IRF-1-/-). The fate of the donor cells was
for 30 rain in 0.1 ml of PBS containing 1% BSA and 0.1% so-
at room temperature and were subsequently incubated on ice
CA) using CellQuest software (Becton Dickinson). Viable lym-
cytometry (FACScalibur ®, Becton Dacklnson & Co., San Jose,
with mAbs [anta-NKl.1 (PK136, PE conjugated), antl-CD3
i.p pI:pC at 1 and 24 h after injecuon of donor cells.
Mice were prepared from 6--8-wk-old C57BL/6 (H-2 b) and C57BL/6 X
Monocytes were removed from IL-12-stimulated spleen MNC after a 24-h
in complete medium (CM; 4-MEM containing 10% FCS, 20
mM Hepes, and 10 µM B-mercaptoethanol) within 48--well tissue
culture plates. Cells were recovered at day 9, washed twice, counted,
grown in CM, labeled with Na251CrO4 (NEN, Boston, MA; 35
Ci/106 cells), and added to wells containing MNC. Superna-
tants from spleen cells cultured in the presence of
were injected subcutane-
ously (6). For experiments involving the injection of F1 cells, C57BL/6 (H-2b) IRF-1+/+ mice were bred with A/J (H-2a)
IRF-1-/- mice (kindly provided by Dr. J. Penninger, Amgen Insti-
tute, Toronto). Mice were housed in specific pathogen-free
conditions, and littermate controls were used within experiments.

### Induction of NK Activity In Vitro.
Mice were injected with 0.1 mg i.v. pI:pC (Pharmacia, Uppsala, Sweden) or 2 X 106 PFU
LCMV (Armstrong strain). After 24 h (pI:pC) or 72 h (LCMV),
spleens were harvested and mononuclear cells (MNC) isolated by
density gradient centrifugation (Lymphocyte M; Cedarlane, On-
tario, Canada).

### Induction of NK Activity In Vivo.
Spleen MNC were cultured in complete medium (CM; α-MEM containing 10% FCS, 20
mM Hepes, and 1 µM B-mercaptoethanol) within 48--well tissue culture plates at a concentration of 106 cells/ml. Wells con-
tained murine rIFN-β (Lee Biomolecular Research, San Diego,
CA) or murine rIL-12 (Genzyme, Cambridge, MA). Plates were
incubated for 72 h (IFN-β) or 24 h (IL-12) at 37°C/5% CO2.

### Evaluation of IFN-γ in IL-12-stimulated Spleen Culture.
Superna-
tants were removed from IL-12-stimulated spleen MNC after a 24-h culture, and IFN-γ was measured by ELISA (Innerset-γ, Genzyme).

### NK Cytotoxicity Assay.
MNC were added to V-bottomed 96-well plates in CM. The NK-sensitive cell line YAC-1 was grown in CM, labeled with Na235CrO4 (NEN, Boston, MA; 35 µCi/106 cells), and added to wells containing dilutions of MNC. Target cell lysis was determined after a 4-h incubation by measure-
tment of 51Cr release using the following formula: percent of spe-
fic lysis = (a - b/c - b) X 100%, where a = test release, b = spontaneous release, and c = release in the presence of 1% SDS. Spontaneous release was always <10%.

### Analysis of IL-2-stimulated Cells.
107 spleen MNC were cultured in CM with 500 U/ml murine rIL-2 in 24--well tissue culture plates. Cells were recovered at day 9, washed twice, counted, and analyzed by flow cytometric analysis.

### Tumor Elimination In Vivo.
Mice were injected subcutane-
ously in the right flank with 106 RMA-S cells in 0.1 ml PBS (8). Mice were monitored for palpable tumors and were killed when tumors reached a diameter of 1 cm.

### Persistence of Potential Donor Cells.
Spleen and LN MNC cells were prepared from 6--8-wk-old C57BL/6 (H-2b) and C57BL/6 X
A/J F1 (H-2a) mice by density gradient centrifugation. After
two washes in CM, the cells were labeled with FITC (Sigma Im-
munocoolies, St. Louis, MO; 30 µg/ml in PBS) for 18 min at
37°C/5% CO2. Excess FITC was removed by centrifugation
through 6% BSA in PBS, and 2 X 107 cells in 0.2 ml PBS were
injected into the lateral tail vein of recipient mice (C57BL/6 X
A/J F1, IRF-1+/+, or IRF-1-/-). The fate of the donor cells was
monitored by flow cytometry of peripheral blood and LN cells.
NK activity was induced in recipient mice by injection of 0.1 mg
i.p. pI:pC at 1 and 24 h after injection of donor cells.

### Flow Cytometric Analysis.
MNC were preincubated with anti-
FcyR mAb (Fc block; Pharmingen, San Diego, CA) for 10 min
at room temperature and were subsequently incubated on ice
with mAbs [anti-NK1.1 (PK136, PE conjugated), anti-CD3
(145-2C11, FITC-conjugated), anti-CD69 (H1.2F3, biotiny-
lated), anti-CD25 (7D4, biotinylated), and anti-CD11a (M17/4,
biotinylated); all mAbs were from Pharmingen (San Diego, CA)]
for 30 min in 0.1 ml of PBS containing 1% BSA and 0.1% sod-
umide. Cells were washed with PBS and analyzed by flow
cytometry (FACScalibur®, Becton Dickinson & Co., San Jose,
CA) using CellQuest software (Becton Dickinson). Viable lympho-
cytes were gated on the basis of forward and side scatter char-
acteristics, and 10,000 gated events were analyzed.

Results

**NK Cytotoxicity in IRF-1-deficient Mice in Response to LCMV Infection and pI:pC Treatment.** The role of IRF-1 in the induction of NK cell cytotoxicity in response to LCMV infection was investigated. 72 h after high dose infection
with 2 X 106 PFU LCMV, significant lysis of the prototypic NK-sensitive cell line YAC-1 was observed with spleen cells from IRF-1+/+ mice, but not with cells from IRF-1-/- mice (Fig. 1 A). Spleen cells from uninfected IRF-1+/+ or IRF-1-/- mice displayed no measurable lytic activity (data not shown). Stimulation of NK lytic activity in response to the type 1 IFN inducer pI:pC was also investigated. 24 h after administration of 0.1 mg i.v. pl:
pC, significant lysis of YAC-1 target cells was clearly ob-
erved in wild-type mice (Fig. 1 B). However, pI:pC-
treated IRF-1-/- mice exhibited markedly reduced lytic activity compared to wild-type mice, confirming the results obtained with LCMV.

**IFN-β-enhanced Cytolytic Activity In Vivo.** To address the possibility that a defect in type 1 IFN production in IRF-1-/- mice was responsible for the lack of cytotoxicity observed
in IRF-1-/- mice, spleen NK cells were incubated in vitro with IFN-β. IFN-β induced a dose-dependent activation of NK-mediated lytic activity in wild-type animals, with a
maximal effect observed in the presence of 1,000 IU/ml
IFN-β (Fig. 2), as reported previously (9). IRF-1-deficient
spleenocytes demonstrated maximal lytic activity at the same
dose of IFN-β, but exhibited greatly reduced lytic activity
compared to wild-type controls.

**IL-12-enhanced Cytolytic Activity In Vitro.** Spleen MNC-
derived from IRF-1+/+ and IRF-1-/- mice were incubated
in the presence of 10 ng/ml IL-12. Significant lysis of the
target cell line by IRF-1+/+ spleen NK cells was induced by
IL-12 (Fig. 3 A), while IL-12 failed to augment the cy-

tolytic activity of IRF-1-deficient NK cells. Analysis of the
supernatants from spleen cells cultured in the presence of

![Figure 1](image-url)
Figure 2. NK-specific lysis of YAC-1 target cells after an in vitro induction of NK activity by IFN-γ. Black bars represent lysis by IRF-1+/− spleen NK cells, and white bars represent lysis by IRF-1−/− spleen NK cells at an E/T ratio of 100:1. Data are shown as mean ± SEM for a single experiment performed in quadruplicate in which cells from two mice of each genotype were pooled, and are representative of two experiments.

10 ng/ml IL-12 revealed a defect in IFN-γ production in IRF-1−/−-derived spleen cells (Fig. 3 B).

IL-2-stimulated Proliferation and Cytolytic Activity In Vitro. Incubation in the presence of 500 U/ml IL-2 for 9 d induce preferential proliferation of NK cells, with NK1.1+/CD3− cells comprising 61.2 ± 1.2% of recovered IRF-1+/−-derived cells, compared to 44.5 ± 3.6% in the case of IRF-1−/−-derived spleen cells (mean ± SEM for three mice in each group). Both IRF-1+/− and IRF-1−/−-derived cells contained approximately equal percentages (2.5-3.0%) of NK1.1+/CD3− cells at the start of the 9-d culture. Calculation of absolute numbers of NK1.1+ and CD3+ cells revealed a more profound deficit in IRF-1−/−-derived NK cell proliferation in response to IL-2 (Fig. 4 B), with a five-fold decrease in viable NK1.1+ cells present after 9 d. The cytolytic activity of day 9 IL-2-stimulated NK cells was evaluated against YAC-1 target cells (Fig. 4 C) and revealed that although IRF-1−/− LAK cells are clearly capable of killing NK cell targets, IRF-1−/−-derived spleen cells have a five-fold lower cytolytic activity (Fig. 4 C). Two mase of each genotype are shown (closed symbols represent heterozygous IRF-1−/− mase, open symbols represent homozygous IRF-1−/− mase), and white bars represent control effector cells. Open symbols represent homozygous IRF-1−/−-derived spleen cells (mean ± SEM for three mice in each group). Analysis of supernatants taken from IL-12-stimulated spleen cell culture (10 ng/ml) revealed a defect in IFN-γ production (B). Black bars represent IFN-γ production by IRF-1+/− mice, and white bars represent IRF-1−/− mice. Data are shown as mean ± SEM of an experiment with two mase in each group performed in triplicate, and are representative of six animals.

NK activity was induced in recipient mice by the injection of pL:pC at the time of donor cell injection and at 24 h after injection. Persistence of FITC-labeled donor cells in the LN of recipient animals was followed by flow cytometry (Fig. 6). While F1 syngeneic donor cells persisted in F1 hosts, C57BL/6-derived parental donor cells (H-2b) were eliminated in F1 IRF-1−/− mice. In contrast, F1 IRF-1−/− mice were unable to eliminate the parental donor cell graft.

Normal Numbers and Surface Markers of IRF-1−/− NK1.1+ NK Cells. The potential role of IRF-1 in the ontogeny and activation of murine NK cells was addressed by flow cytometric analysis of spleen MNC that were obtained from pL:pC-treated mice (Fig. 7). No significant difference in the number of NK1.1+/CD3− cells was evident between heterozygous and homozygous IRF-1−/− mice, and the number of CD3+/NK1.1+ cells expressing the early activation marker CD69 was similar in both groups of mice. Comparable numbers of CD3+/NK1.1+ cells also expressed the α chain of the IL-2 receptor (CD25) and CD11a, the α chain of LFA-1, in both heterozygous and homozygous IRF-1−/− mice (data not shown).

Discussion

From the data presented in this study, it is clear that IRF-1 plays an essential role in the augmented lytic activity of murine NK cells after viral infection, stimulation with type I IFNs, or stimulation with IL-12. Flow cytometric analysis of spleen MNC from heterozygous and homozygous IRF-1−/− mice indicates that IRF-1 does not play a role in the ontogeny of NK cells, and that the defect seen in NK cell-mediated cytotoxicity is not caused by a
Figure 4. IL-2-stimulated proliferation and augmented killing of NK cells in IRF-1-deficient mice. A: FACS analysis of NK1.1+ and CD3+ cells at day 9 (B) were determined from total viable cell counts and the percent of cells obtained from FACS analysis. Solid bars represent cells from in vitro cultured cells, while open bars represent cells from mice injected with IL-2. B: Tumor incidence (%) of IRF-1+/- and IRF-1-/- mice injected with RMA-S cells. C: Kaplan-Meier survival curves of IRF-1+/- and IRF-1-/- mice injected with RMA-S cells.

Figure 5. Elimination of MHC class I-negative syngeneic tumor is impaired in IRF-1-deficient mice. IRF-1+/+ (closed symbols) and IRF-1-/- (open symbols) mice were injected subcutaneously with 10^6 RMA-S cells and monitored for palpable tumors. Five age- and sex-matched mice were analyzed in each group.

IL-12 was first identified as an inducer of NK cell-mediated cytotoxicity (14), presumably through the induction of genes involved in target cell lysis, such as perforin or granzyme B (15). Another important function of IL-12 is the induction of IFN-γ production in resting and activated NK cells (16), a key step in the innate response to acute infection. In this study, we report that IRF-1-deficient NK cells are incapable of responding appropriately to IL-12 stimulation in vitro, since both enhanced lytic activity and IFN-γ production are defective in splenocytes derived from IRF-1-/- mice. Our data suggest that IL-12-mediated signaling itself is defective in the NK cells of IRF-1-deficient mice, correlating with a recent report that IL-12-deficient mice exhibit a defect in both NK cell cytolytic activity and IFN-γ production (17). IL-12 has also been implicated as a central mediator of acute GVHD in BDF1 mice injected with B6 donor splenocytes. Neutralization of endogenous IL-12 moderated acute GVHD and resulted in decreased splenic NK activity and IFN-γ production (18). We therefore postulate that the defective rejection of semiallogeneic, hematopoietic donor cells by IRF-1-/- mice is, at least in part, the result of aberrant IL-12-mediated signaling.

The functional response of NK cells to IL-2 stimulation in vitro was also found to be disrupted in IRF-1 knockout mice. Both proliferation and augmentation of lytic activity of IL-2-stimulated NK cells were defective, although the partial reduction in lytic activity seen in IRF-1-/- compared to IRF-1+/+ controls was not as profound as that seen after activation with type I IFNs or IL-12. It appears, therefore, that IL-2-mediated effects on NK cell function are not fully dependent on IRF-1.
The expression of some genes is critically dependent on IRF-1, for example, inducible nitric oxide synthase (iNOS) or guanylate-binding protein (19). It has recently been suggested that NO may play a role in human NK cell–mediated cytotoxicity (20), and given the dependence of iNOS on IRF-1 for induction, the possibility that the defect in NK cell–mediated cytotoxicity in IRF-1--/ mice is caused by the reduced production of NO was addressed in our laboratory. The nonspecific NO synthase inhibitor N-monomethyl-L-arginine and the iNOS-specific inhibitor aminatinguancine exhibited no inhibitory effect (at doses ranging from 10 nM to 1 mM) on pI:pC-induced, NK-mediated killing of YAC-1 target cells in vitro (data not shown). This suggests that NO does not play a role in NK cell–mediated cytotoxicity, and supports the finding that perforin is predominately responsible for NK cell–mediated cytotoxicity (21, 22).

The expression of many IFN-inducible genes is mediated through IRF-1–independent pathways, including double-stranded RNA–dependent protein kinase and 2′-5′ oligoadenylate synthetase (6). It is possible that the residual NK-mediated lytic activity of IRF-1--/ cells observed in our experiments may be mediated through other transcription factors. Candidates include IFN-γ–inducible γ-activated factor, via its association with the IFN-γ activation site, or IFN-stimulated gene factor 3 (ISGF3), which shares overlapping sequence recognition with IRF-1, and may cooperate with IRF-1 in the induction of type I IFN-inducible genes in a nonredundant fashion (23). Although NK cells both produce and respond to IFN-γ, experiments using IFN-γ-deficient mice show that enhanced splenic NK cell activity from pI:pC–treated mice is independent of type II IFN, and that induction of type I IFNs can at least compensate for an absence of IFN-γ (24).

The data obtained in our in vivo studies of hybrid resistance to a hematopoietic graft, as well as the rejection of an MHC class I–negative tumor cell from a peripheral site of injection, confirm the in vitro data showing a lack of NK cell–mediated cytotoxicity in IRF-1--/ mice. While type I IFNs were induced in the in vitro models of NK activity by biological response modifiers, the rejection of allogeneic grafts and MHC class I–negative tumor cells are models for which the role of endogenous IFNs remains to be elucidated. Our data does, at least indirectly, support findings that IL-12 plays a prominent role in the acute rejection of hematopoietic allografts and MHC class I–negative cells in the mouse.

In conclusion, the absence of the transcription factor IRF-1 leads to a defect in NK cell cytolytic activity induced in response to LCMV infection, pI:pC, IFN-β, IL-12, or IL-2, which is not the result of reduced NK cell number, or of NK cell activation as assessed by the expression of the activation markers CD69 and CD25. In addition, MHC class I–deficient tumor cells are not eliminated, and clearance of parental donor cells is impaired in IRF-1–deficient mice. IRF-1 is therefore an essential mediator of normal NK cell function in the mouse.

The authors would like to thank Drs. Thomas Kündig and Martin Bachmann for the administration of LCMV and the gift of RMA-S cells, Julia Potter for genotyping mice, John Shannon for the gift of YAC-1 cells, Drs. David Ferrick, Richard G. Miller, and Sam Kung for helpful discussions, and Dr. Mary Saunders.
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