Deficiency in the Transcription Factor Interferon Regulatory Factor (IRF)-2 Leads to Severely Compromised Development of Natural Killer and T Helper Type 1 Cells

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Abstract

Interferon (IFN) regulatory factor (IRF)-2 was originally described as an antagonist of IRF-1-mediated transcriptionsal regulation of IFN-inducible genes. IRF-1−/−/2 mice exhibit defective T helper type 1 (Th1) cell differentiation. We have used experimental leishmaniasis to show that, like IRF-1−/− mice, IRF-2−/− mice are susceptible to Leishmania major infection due to a defect in Th1 differentiation. Natural killer (NK) cell development is compromised in both IRF-1−/− and IRF-2−/− mice, but the underlying mechanism differs. NK (but not NK1T) cell numbers are decreased in IRF-2−/− mice, and the NK cells that are present are immature in phenotype. Therefore, like IRF-1, IRF-2 is required for normal generation of Th1 responses and for NK cell development in vivo. In this particular circumstance the absence of IRF-2 cannot be compensated for by the presence of IRF-1 alone. Mechanistically, IRF-2 may act as a functional agonist rather than antagonist of IRF-1 for some, but not all, IFN-stimulated regulatory element (ISRE)-responsive genes.

Key words: interferon regulatory factor • Th1 • natural killer cells • Leishmania • interleukin 15

Introduction

The IFN regulatory factor (IRF) family includes IRF-1 through IRF-7, IFN-stimulated gene factor 3γ (ISGF3γ), and IFN consensus sequence-binding protein (ICSBP) (1). Members of the IRF family were originally identified by their ability to bind DNA sequences common to the promoters of the IFN-α/β genes (2). IRF family members also bind to IFN-stimulated regulatory elements (ISREs) found in promoters of IFN-inducible genes, such as inducible nitric oxide synthase (iNOS) (3). Studies of wild-type embryonic cells and fibroblasts (2, 4) have suggested that IRF-1 and IRF-2 compete for binding at the same DNA locus and can act as mutual antagonists. Although IRF-1 induces expression of IFN-α/β, IRF-2 represses it (4, 5). Similarly, although IRF-1 has antioncogenic activity, IRF-2 induces tumorigenicity in embryonic 3T3 cells (6). Mutual antagonism is also suggested by the phenotypes of mice with a targeted disruption of either the IRF-1 or the IRF-2 gene (7, 8). Type I IFN induction in response to poly(I):poly(C) is reduced in IRF-1−/− fibro-
blasts, but upregulated in IR F-2-/- fibroblasts after infection with New castle disease virus (7, 8). However, in contrast to its repressive effects on IFN-related genes, IR F-2 activates the transcription of histone genes and the vascular cell adhesion molecule (VCAM)-1 gene (11–14). Interestingly, nuclear extracts of spleen cells from ICSPB-/- mice contain no detectable IR F-2 activity (15), implying a role for ICSPB in the posttranscriptional/translational modification of IR F-2.

ISR E consensus binding sites occur in the promoters of the IL-4 and IL-12 genes (16, 17), genes that are critical for the differentiation of naïve T cells into Th2 or Th1 cells, respectively (18–20). IR F-1/-/- mice display a defect in Th1 differentiation, primarily due to reduced IL-12 production (21, 22). Deficiency for ICSPB, which can bind to IR F-1, leads to a similar decrease in IL-12 synthesis and impaired Th1 cell differentiation (23, 24). It has recently been reported that IL-12 production is also dysregulated in IR F-2-deficient macrophages (25). We thus decided to test the effects on Th differentiation of a null mutation in IR F-2, the putative repressor of IR F-1 and an alternative partner of ICSPB.

M urine cutaneous leishmaniasis, induced by subcutaneous inoculation of the protozoan parasite Leishmania major, is a useful experimental model in which to study Th differentiation. L. major infection is self-healing in resistant mice (e.g., C57BL/6) because of the expansion of macrophage-activating Th1 cells, but is lethal in susceptible mice (e.g., BALB/c) due to the expansion of macrophage-deactivating Th2 cells (26). We have used this model to demonstrate that IR F-2 is required not only for IL-12 production but also for the development of Th1 cells and NK cells, a dominant IFN-γ-secreting and IL-12-inducing cell population.

Materials and Methods

M ouse M ice were housed in specific pathogen-free facilities according to the ethical and institutional guidelines of the Ontario Cancer Institute and the Institut für Klinische Mikrobiologie und Immunologie, Universität Erlangen. IR F-2-/-, IR F-2-/-/-, and IR F-2-/-/- littermates (7), backcrossed for seven generations to C57BL/6 mice, were used in all experiments. Control BALB/c, C57BL/6, and 129/SvJ mice were purchased from The Jackson Laboratory.

Infection of Mice with L. major and Monitoring of the Disease. M ice (three to four per group) were infected in the right hind footpad with 2 x 10⁶ stationary phase promastigotes of L. major strain MHOM/IL/81/EBN1 (27) and the increase in footpad thickness (expressed as a percentage) was calculated (22). BALB/c mice had to be killed by week 5 due to severe ulcerations. Parasite burden was determined by limiting dilution analysis as described previously (28). The number of parasites per organ or per cell number plated was determined for each mouse.

Determination of Cytokines by ELISA. At 4 and 7 wk after infection of mice with L. major, right popliteal LN s were removed and single cell suspensions were made. Cells were restimulated in vitro with or without L. major antigens, as described (22). After 48 h, culture supernatants (SN s) were harvested and IL-4 and IFN-γ levels were determined using ELISA kits (BD PharMingen).

Cell Transfer Experiments. Purification of C57BL/6 wild-type and recombination activating gene (RAG)-1/-/- B cells was performed as described previously (22) using the MACS system (Miltenyi Biotec). The resulting cell population contained >90% B220⁺ cells and <0.5% CD4⁺ and CD8⁻ T cells.

To obtain purified CD4⁺ cells, spleens and LN s from groups of three IR F-2-/- or IR F-2-/-/- mice were pooled and CD4⁺ T cells were isolated using MACS (22). The resulting population consisted of >90% (in the case of IR F-2-/-) and >98% (for IR F-2-/-/-) CD4⁺ cells. Contamination with CD8⁻ cells was <0.2%. RAG-1/-/- mice (three per group) were reconstituted intraperitoneally with 11 x 10⁶ B cells with or without 6 x 10⁶ IR F-2-/- or IR F-2-/-/- CD4⁺ T cells. On the same day, the reconstituted mice were infected with L. major. 6 wk after cell transfer, mice were killed and cytokine production as well as splenic parasite burdens were determined.

In Vitro Differentiation of CD4⁺ T Cells. Pooled single cell suspensions were prepared from spleens, peripheral LN s, or visceral LN s of IR F-2-/- or IR F-2-/-/- mice. CD4⁺ CD62L⁻ T cells were isolated using anti-CD4-FITC (Dianova), the anti-FITC multisort kit (Miltenyi Biotec), and anti-CD62L antibodies conjugated to magnetic beads (Miltenyi Biotec). The purified cell preparation contained 91–99% CD4⁺ CD62L⁻ T cells, referred to as “naïve T cells.” For one experiment, CD4⁺ CD62L⁻ T cells were further purified (>99%) by FACSC® sorting on an EPICS ELITE machine (Coulter). Purified T cells were plated at 10⁶ in 1 ml Click’s medium in 24-well plates previously coated with 5 µg/ml anti-CD3 mAb (29). Th1 differentiation was induced by the addition of IL-12p70 (1 ng/ml; BD PharMingen) plus anti-IL-4 (10% SN s of hybridoma 11B11 [30]), whereas Th2 differentiation was induced with IL-4 (10% SN s of X63Ag8-653-653-MLC-4 cells [31]) plus neutralizing anti-IL-12 antisera (10 µg/ml; provided by Dr. M. Gately, Knoll Pharmaceutical Co., North M ount O live, N J.). After 96 h, half of the SN was replaced with fresh medium containing IL-2 (10% SN s of X63Ag8-653-653-MLC-4 cells [31]) and the cells were transferred to wells without anti-CD3. After an additional 48 h, the cells were harvested, washed, and plated (10⁶/well) in 200 µl medium in 96-well plates, with or without anti-CD3. After 24 h, SN s were tested for IL-4 or IFN-γ production as described above.

Quantitative Real Time Reverse Transcription PCR by TaqMan Analysis. IR F-2-/- and IR F-2-/-/- mice were infected with L. major as described above. 10 d later, popliteal LN s (LN s) were prepared and total RNA was purified using the RNeasy kit (Qiagen). Primers (from MWG Biotech Ebersberg) and probes (Eurogentec) were as follows. β-actin: 5’ CACCCCACT-GTCCCCATCTATGA; 3’ GATGCCACAGGATTCCATG, and 5′-actin: 5′-mGTCCTGCCAGGAGG; 3′ CAGTTTGGCCAGGGTCATTCCA. IL-12p40: 5′ GTGGTTTTAGAGGAGG; 3′ CTCCACGTGCCATCTATGA; 3′ GATGCCACAGGATTCCATG. IL-12p35: 5′ GCAGTTTGGCCAGGGTCATTCCA. IL-12p40: 5′ GTGGTTTTAGAGGAGG; 3′ CGTTTGGCCAGGGTCATTCCA. IL-12p35: 5′ CAGTTTGGCCAGGGTCATTCCA. IL-12p40: 5′ GTGGTTTTAGAGGAGG; 3′ CGTTTGGCCAGGGTCATTCCA. IL-12p35: 5′ CCACCCTTGCCCTCCTAAAC; 3′ GATGCCACAGGATTCCATG. IL-12p35: 5′ CCACCCTTGCCCTCCTAAAC; 3′ GATGCCACAGGATTCCATG. The one tube reverse transcription (RT)-PCR reaction (25 µl volume) consisted of 200 nM each primer, 100 nM probe, 300 µM dNTP s, 3.5 mM MgCl₂, 2 µl RNA, 2.5 U Tth polymerase (all from PerkinElmer), and 1 × PCR buffer (50 mM bicarbonate, 125 mM potassium acetate, 10 µM EDTA, 8% glycerol, pH 8.2). In the presence of magnesium, Tth polymerase acts as reverse tran-
SNs were assayed for nitrite (NO$_2$) and murine recombinant IFN-$
$-gated on the basis of forward and side scatter, and 10,000 events were analyzed by flow cytometry on a FACSCalibur™ using CELLQuest™ software (BD PharMingen). For detection of biotinylated antibody, Ly9.1 (PK136), anti–pan-NK–FITC (DX5), anti-TCR-$\alpha$–allophycocyanin (APC) (H57-597), anti-IL-2–biotin (30C7), anti-B220–FITC (RA3-6B2), anti-CD44–PE (IM7), and processed for cell cycle analysis using the manufacturer’s instructions. The percentage of CD44$^+$CD8$^+$ cells in S phase was quantified by flow cytometry (FACSCalibur™ using CellQuest™ software).

**Results**

Enhanced Susceptibility of IRF-2$^{-/-}$ Mice to L. major Infection. To determine the role of IRF-2 in protection against infection with L. major, the course of leishmaniasis was examined in wild-type C57BL/6 mice and in IRF-2$^{-/-}$ and IRF-2$^{+/+}$ mice of the resistant C57BL/6 background. Wild-type C57BL/6 mice developed a temporary swelling of the footpad which healed by week 7, as did IRF-2$^{+/+}$ mice (Fig. 1 A). In contrast, IRF-2$^{-/-}$ mice developed a progressive increase in footpad thickness and ulcerations similar to, but not as prominent as, those in susceptible BALB/c mice (Fig. 1 A) or IRF-2$^{+/+}$ mice (22).

To determine whether the increased footpad swelling in IRF-2$^{-/-}$ mice reflected a higher systemic parasite load, parasite numbers in spleens, lesions, and lesion-draining lymph nodes were measured. (A) Time-dependent increase in lesion size after L. major infection in BALB/c (C57BL/6), IRF-2$^{+/+}$, and IRF-2$^{-/-}$ mice. Mice were infected with L. major as described in Materials and Methods, and the increase in footpad thickness (%) was calculated at the indicated time points. Data are shown as mean ± SD of four mice per group, and are representative of three independent experiments. (B) Protection of RAG-1$^{-/-}$ mice from L. major infection by transfer of IRF-2$^{+/+}$ CD4$^+$ T cells. RAG-1$^{-/-}$ mice received either no cells or were injected intravenously with B cells from IRF-2$^{+/+}$ mice without (B), or with (B + IRF-2$^{+/+}$CD4) or B + IRF-2$^{+/+}$CD4$^+$ T cells from IRF-2$^{+/+}$ or IRF-2$^{-/-}$ mice, respectively. The same day, all mice were infected with L. major and lesion sizes were monitored as described above. Data shown are the mean ± SD of three mice per group.
Spleen mice exhibited a strong Th1 response to *C.57BL/6* spleens, a burden equal to that observed in mice taken 4 wk after infection with *LmAg* by total popliteal LNCs of individual IRF-2 g

Amounts of IFN-γ and IL-4 secreted in response to *LmAg* culture SNs were tested 48 h later for IL-4 and IFN-γ production. IRF-2/−/− mice (four mice per group) 4 and 7 wk after infection *C.57BL/6* and IRF-2/−/− mice were infected subcutaneously with *L. major* antigens was also determined (Table III). IRF-2/−/−CD4+ T cells produced slightly lower total amounts of both IL-4 and IFN-γ compared with IRF-2+/−CD4+ T cells. However, the Th1-indicative IFN-γ/IL-4 ratio was even increased compared with that of IRF-2+/−CD4+ cells. Thus, in mice bearing functional IRF-2+/−/APCs, the differentiation in vivo of IRF-2−/−CD4+ T cells into Th1 cells was essentially normal. These experiments indicate that the inability of IRF-

### Table I. Clearance of *Leishmania* Is Defective in IRF-2−/− Mice

| Organ          | No. of weeks after infection | C57BL/6/+/+ | IRF-2−/− | IRF-2−/− |
|----------------|-----------------------------|-------------|----------|----------|
| Footpad        | 4                           | 5.0 × 10^5 ± 2.5 × 10^5 | 2.5 × 10^6 ± 1.7 × 10^5 | 2.5 × 10^7 ± 1.5 × 10^2 |
|                | 7                           | 7.8 × 10^5 ± 7.3 × 10^5 | 1.600 ± 800 | 4.0 × 10^6 ± 3.5 × 10^4 |
| LN             | 4                           | 1,900 ± 400 | 1,200 ± 500 | 1.6 × 10^4 ± 1.1 × 10^4 |
|                | 7                           | 35 ± 13     | 108 ± 64 | 1.1 × 10^6 ± 0 |
| Spleen         | 4                           | 1 ± 1       | 10 ± 8    | 1,500 ± 1,200 |
|                | 7                           | 2 ± 1       | 240 ± 96 | 1.5 × 10^6 ± 3.0 × 10^5 |

Parasite burden in the infected footpad, popliteal LN draining the infected footpad, or spleen of individual wild-type C57BL/6, IRF-2−/−, or IRF-2−/− mice (four mice per group) 4 and 7 wk after *L. major* infection. Results are given as the number of parasites in the footpad, LN, and spleen of the respective mice. Data represent the mean ± SD of all mice in one group and are representative of three separate experiments.

| Organ          | No. of weeks after infection | C57BL/6/+/+ | IRF-2−/− | IRF-2−/− |
|----------------|-----------------------------|-------------|----------|----------|
| Footpad        | 4                           | 5.0 × 10^5 ± 2.5 × 10^5 | 2.5 × 10^6 ± 1.7 × 10^5 | 2.5 × 10^7 ± 1.5 × 10^2 |
|                | 7                           | 7.8 × 10^5 ± 7.3 × 10^5 | 1.600 ± 800 | 4.0 × 10^6 ± 3.5 × 10^4 |
| LN             | 4                           | 1,900 ± 400 | 1,200 ± 500 | 1.6 × 10^4 ± 1.1 × 10^4 |
|                | 7                           | 35 ± 13     | 108 ± 64 | 1.1 × 10^6 ± 0 |
| Spleen         | 4                           | 1 ± 1       | 10 ± 8    | 1,500 ± 1,200 |
|                | 7                           | 2 ± 1       | 240 ± 96 | 1.5 × 10^6 ± 3.0 × 10^5 |

Parasite burden in the infected footpad, popliteal LN draining the infected footpad, or spleen of individual wild-type C57BL/6, IRF-2−/−, or IRF-2−/− mice (four mice per group) 4 and 7 wk after *L. major* infection. Results are given as the number of parasites in the footpad, LN, and spleen of the respective mice. Data represent the mean ± SD of all mice in one group and are representative of three separate experiments.

Table II. Amounts of IL-4 and IFN-γ (in pg/ml) Secreted In Vitro in Response to *L. major* Antigens

|              | IRF-2−/− | IRF-2−/− |
|--------------|----------|----------|
|              | −LmAg    | +LmAg    | −LmAg    | +LmAg    |
| IL-4         | <10      | <10      | <10      | 40 ± 9   |
| IFN-γ        | 206 ± 98 | 2,790 ± 1,438 | 75 ± 11 | 88 ± 19 |

Amounts of IFN-γ and IL-4 secreted in response to *L. major* antigens (LmAg) by total popliteal LNCs of individual IRF-2+/− and IRF-2−/− mice taken 4 wk after infection with *L. major*. Data are shown as mean ± SD for three mice in each respective group and are representative of three independent experiments.
Table III. Cytokine Production after In Vivo Transfer of IRF-2\textsuperscript{−/−} and IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells into RAG-1\textsuperscript{−/−} Mice

| Transferred cells        | -                         | IRF-2\textsuperscript{+/-} B cells | IRF-2\textsuperscript{−/−} B cells +IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} | IRF-2\textsuperscript{−/−} B cells +IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} |
|-------------------------|----------------------------|-------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Parasite burden         | $3.0 \times 10^7 \pm 2.7 \times 10^7$ | $7.2 \times 10^6 \pm 3.6 \times 10^6$ | $8.7 \times 10^4 \pm 1.3 \times 10^4$                                           | $4.0 \times 10^4 \pm 1.3 \times 10^4$                                           |
| | In vitro stimulus       | -LmAg                      | +LmAg                               | -LmAg                                                                            | +LmAg                                                                            |
|                          | IFN-$\gamma$ (pg/ml)       | $<40$                               | $<40$                                                                             | $80 \pm 0$                                                                       | $3.500 \pm 70$                                                               | $<40$ | $2.000 \pm 0$       |
|                          | IL-4 (pg/ml)               | $<5$                                | $<5$                                                                             | $55 \pm 35$                                                                      | $375 \pm 250$                                                               | $<5$  | $50 \pm 14$         |

6 wk after the start of the experiment, splenocytes were prepared from L. major-infected RAG-1\textsuperscript{−/−} mice (three mice per group) as described in Materials and Methods. Parasite burden (expressed as the number of leishmania per spleen) and cytokine production in pooled cells from each group were determined as described in Materials and Methods. Data are shown as mean ± SD of triplicate determinations.

IRF-2\textsuperscript{−/−} mice to develop a Th1 response is not a result of a primary defect in CD4\textsuperscript{+} T cells.

The capacity of CD4\textsuperscript{+} T cells of IRF-2\textsuperscript{−/−} and IRF-2\textsuperscript{−/−} mice to differentiate into Th1 cells was also compared in vitro. Naive CD4\textsuperscript{+}CD62L\textsuperscript{+} T cells of both genotypes were cultured either under neutral conditions or in a cytokine milieu favoring the differentiation of either Th1 or Th2 cells. After resting and restimulation, culture SNs were tested for the presence of IL-4 or IFN-$\gamma$. As shown in Table IV, both IRF-2\textsuperscript{−/−} and IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells that were primed so as to promote a Th1 response indeed secreted high amounts of IFN-$\gamma$ and no IL-4, confirming that Th cells of IRF-2\textsuperscript{−/−} mice have no inherent impediment to Th1 differentiation. In addition, Th2-primed IRF-2\textsuperscript{−/−} and IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells secreted high amounts of IL-4 and lower amounts of IFN-$\gamma$, showing that a lack of IRF-2 does not preclude IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells from adopting a Th2 phenotype. Interestingly, however, Th2-primed IRF-2\textsuperscript{+/-} CD4\textsuperscript{+} T cells produced substantially more IFN-$\gamma$ than IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells. Such a difference was even more obvious between IRF-2\textsuperscript{−/−} and IRF-2\textsuperscript{+/-} CD4\textsuperscript{+} T cells primed under neutral conditions (anti-CD3 alone). In the absence of a strong stimulus towards either Th1 or Th2 development, IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells (like wild-type CD4\textsuperscript{+} T cells) spontaneously developed into Th1 cells, although they produced less IFN-$\gamma$ than cells stimulated in the presence of IL-12. In contrast, neutrally primed IRF-2\textsuperscript{+/-} CD4\textsuperscript{+} T cells failed to develop into either Th1 or Th2 cells, a finding reproduced in four independent experiments using FACS\textsuperscript{®}-sorted cells of purity >99%. The absence of Th1/Th2 cells was not caused by apoptosis of IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells upon restimulation, because (a) IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells proliferated to the same extent as IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells under neutral conditions, and (b) IRF-2\textsuperscript{−/−} CD4\textsuperscript{+} T cells retained the capacity to differentiate into IFN-$\gamma$- or IL-4-producing cells after sec-

Table IV. In Vitro Differentiation of CD4\textsuperscript{+} IRF-2\textsuperscript{+/-} and IRF-2\textsuperscript{−/−} T Cells

| Secondary stimulus | IFN-$\gamma$ | IL-4 | IFN-$\gamma$ | IL-4 |
|-------------------|-------------|------|-------------|------|
| Primary stimulus  |             |      |             |      |
| Anti-CD3          | $<0.016$  | 6    | $<0.0075$   | 65   |
| Anti-CD3 + IL-4 + anti-IL-12 | $<0.016$ | 13   | $<0.0075$   | 65   |
| Anti-CD3 + IL-12 + anti-IL-4 | 1        | 260  | $<0.0075$   | 65   |

Pooled naive CD4\textsuperscript{+} T cells were purified from spleens and LN\s of IRF-2\textsuperscript{−/−} and control IRF-2\textsuperscript{+/-} mice. The cells were stimulated with immobilized anti-CD3 and the indicated (primary stimulus) cytokines and antibody for 96 h, then washed and transferred to wells without anti-CD3. After an additional 48 h, the cells were washed and restimulated in the presence or absence of anti-CD3 (secondary stimulus). SNs were harvested after 24 h and analyzed in triplicate for IFN-$\gamma$ and IL-4 production (in ng/ml) by ELISA. In all cases, the SD of these determinations was <10%.
ondary stimulation in the presence of IL-12 or IL-4 (data not shown). In addition, IRF-2^{-/-} and IRF-2^{+/+} CD4^{+} T cells expressed equal levels of CD3 (not shown). These data suggest that, in a C57BL/6 background, naive CD4^{+} T cells exhibit a natural bias towards the development of a Th1 response, but that this Th1 bias is absent in IRF-2^{-/-} CD4^{+} T cells.

Reduced IL-12 but Normal NO Production in IRF-2^{-/-} Mice. In IRF-1^{-/-} mice, defective IL-12p70 production was identified as the likely reason for the bias towards the Th2 phenotype (21, 22), prompting us to compare IL-12 production by IRF-2^{-/-}, IRF-2^{+/+}, and IRF-1^{-/-} PEC Mφ stimulated in vitro with LPS and IFN-γ. A marked reduction in IL-12p70 produced by IRF-2^{-/-} PEC Mφ compared with IRF-2^{+/+} PEC Mφ was observed, particularly at limiting concentrations of in vitro stimuli (Fig. 2 A). However, the defect in IL-12 production was not as profound as that observed in IRF-1^{-/-} PEC Mφ, confirming recently published data (25).

Production of NO is critical for clearance of L. major (36). Despite their reduced IL-12 secretion, stimulated IRF-2^{-/-} PEC Mφ produced amounts of NO greater than (in the absence of exogenous IFN-γ) or equal to (in the presence of exogenous IFN-γ) those produced by IRF-2^{+/+} PEC Mφ (Fig. 2 B). IRF-1^{-/-} PEC Mφ failed to produce any NO in response to these stimuli. Flow cytometric analyses demonstrated that PEC Mφ from IRF-2^{-/-}, IRF-2^{+/+}, and IRF-1^{-/-} mice had identical forward/side scatter characteristics and stained equally for Mac-1 and F4/80^{+}. Thus, apart from their defect in IL-12 production, IRF-2^{-/-} PEC Mφ were very similar in gross phenotype to IRF-2^{+/+} PEC Mφ and clearly functional as assessed by NO release. Therefore, the enhanced susceptibility to L. major in IRF-2^{-/-} mice is not due to reduced NO secretion. Normal NO production in the absence of IRF-2 (but not IRF-1) also implies distinct roles for IRF-1 and IRF-2 in the regulation of at least some inducible genes.

To demonstrate defective IL-12 production in the absence of IRF-2^{-/-} in vivo, IL-12 production by popliteal LNCs during L. major infection was quantified. 10 d after infection, total RNA was purified from popliteal LNCs and quantitative RT-PCR was performed to detect expression of the IL-12 p35 and p40 genes (Fig. 2 C). Approximately equal amounts of p35 and p40 mRNAs could be detected in LNCs from uninfected IRF-2^{+/+} and IRF-2^{-/-} mice. In LNCs of L. major-infected mice, the levels of p35 mRNA (the constitutive IL-12 component) were still comparable but there was a significant reduction in p40 mRNA (the inducible component) in IRF-2^{-/-} LNCs compared with IRF-2^{+/+} LNCs, likely accounting for the defective IL-12 production in IRF-2^{-/-} mice in vivo.

Profound Reduction in NK Cell Numbers in IRF-2^{-/-} Mice. Although Th1 cells are the major players in L. major clearance, NK cells also have an important role (37, 38). We thus investigated whether, like IRF-1^{-/-} mice, IRF-2^{-/-} mice exhibit defects in NK cell development and/or function. To assess total NK function, poly(I):poly(C),

Figure 2. Defective IL-12p70 production but normal NO secretion by IRF-2^{-/-} PEC Mφ. (A) IL-12p70 production by PEC Mφ in response to LPS, with or without increasing concentrations of IFN-γ, as determined by ELISA. (B) NO production by the PEC Mφ in A as assessed by measurement of NO_{2}^{-}. Results for A and B are representative of two experiments each and are depicted as the mean ± SD of triplicate samples from two mice per group. ND, not detectable. (C) Expression of IL-12 p35 and p40 genes. Total mRNA from popliteal LNCs of IRF-2^{-/-} and IRF-2^{+/+} mice, either infected with L. major 10 d earlier or uninfected, was prepared from three mice per group. Quantitative RT-PCR to detect p35 and p40 mRNA was performed (see Materials and Methods). The values obtained for uninfected IRF-2^{-/-} mice were arbitrarily set to one, and the data shown give the number of fold increase in p35 and p40 expression in IRF-2^{-/-} mice relative to these values. Values shown are mean ± SD for three mice per group. One experiment representative of two independent trials is shown.
which induces type I IFN and activates NK cells, was injected intravenously into IR F-2i/− and IR F-2i/− mice. 1 d later, splenic mononuclear cells were harvested and tested in vitro for lytic activity against the NK target cell line YAC-1 (Fig. 3 A). IR F-2i/− splenocytes displayed a large decrease in NK activity compared with IR F-2i/− splenocytes, indicating an overall deficit in NK function in this organ. A strong defect in NK cell activity was also noted in vivo in a tumor rejection model using the NK-sensitive cell line RMA-S (data not shown).

To establish if this decreased NK activity correlated with a reduction in NK cell numbers, the proportion of cells carrying the NK cell marker NK1.1, with or without TCR-α/β, was determined in several tissue compartments of IR F-2i/−, IR F-2i/−, and IR F-2i/− mice. In spleen and liver (Fig. 3 B), as well as in peripheral blood (data not shown), the percentage of NK1.1+TCR-α/β cells was dramatically decreased in IR F-2i/− mice compared with IR F-2i/− and IR F-2i/− mice. Strikingly, approximately equal numbers of NK1.1+TCR-α/β+ T (NK1+ T) cells were observed in all three genotypes.

In contrast to the periphery, the proportion of NK1.1+ TCR-α/β− cells in the BM of IR F-2i/− mice was only slightly reduced compared with IR F-2i/− controls (2.82 ± 1.17 vs. 2.01 ± 0.95% in IR F-2i/− and IR F-2i/−, respectively, mean percentage of viable lymphocytes gated ± SD for 11 mice). Thus, the lack of functional NK activity both in vivo and in vitro correlates well with the profound reduction in NK cell numbers in the periphery, but not the BM, of IR F-2i/− mice.

Ablated NK Cell Development in the Absence of IRF-2. The development of NK cells requires interaction between NK progenitors and the BM microenvironment (39). To identify cell populations affected by a lack of IRF-2, BM cells from IR F-2i/− mice in a C57BL/6 background were transferred into irradiated, H-2-compatible 129/SvJ (129) mice. The expression of Ly9.1, a marker expressed on lymphocytes of 129J (Ly9.1+, top left) or IRF-2i/− mice analyzed in parentheses. Percentages of c-kit+NK1.1+CD19− cells were 16.7 ± 2.0 (3), 22.9 ± 3.0 (4), and 69.9 ±
6.1 (5) in IRF-2+/+, IRF-2−/−, and IRF-2−−/− mice, respectively (mean percent ± SD). The surface expression of these markers reconfirms our observation that NK cell development is retarded in IRF-2−−/− mice.

Failure of IL-15 to Support NK Development in IRF-2−−/− BM. The pivotal role of IL-15 in the differentiation, survival, and activation of NK cells (for a review, see reference 40), and the defect in IL-15 production observed in IRF-1−/− mice (41, 42), prompted us to examine IL-15 expression in IRF-2−−/− mice. Expression of IL-15 mRNA species was compared in total BM isolated from IRF-2+/+ and IRF-2−/− mice. IL-15 mRNA was found to be present in greater abundance in IRF-2−/− BM stimulated with LPS plus IFN-γ than in stimulated IRF-2+/+ BM (Fig. 5 A). In conjunction with our BM chimera results, these data indicate that IL-15−/− BM is indeed capable of producing physiologically relevant amounts of IL-15.

We next investigated the ability of IL-15 to support IRF-2−−/− NK cell differentiation and proliferation in vitro. Culture of IRF-2+/+ BM in IL-15 for 7 d resulted in the generation of relatively pure NK cells (~90% NK1.1+ TCR-α/β−, data not shown). After 7 d culture in IL-15, IRF-2−/− BM was also able to generate NK cells (~90% NK1.1+ TCR-α/β−, data not shown) but the yield was markedly reduced compared with IRF-2+/+ BM (Fig. 5 B). In a control experiment (not shown), IL-15 was able to rescue NK cell development in IRF-1−/− BM, confirming previous reports (41, 42). From this data it can be concluded that IRF-1 is clearly not able to fully compensate for a lack of IRF-2 in IL-15-induced NK differentiation, suggesting that the defect in NK development in IRF-2−/− mice differs significantly from that of IRF-1−/− mice.

Surface expression of the IL-2R β chain is thought to be essential for the maturation of NK cell progenitors, conferring responsiveness to IL-15 with the subsequent acquisition of proliferative and lytic potential (43). Both IRF-2+/+ and IRF-2−/− BM NK cells displayed equivalent surface expression of IL-2R β (data not shown), ruling out a defect in IL-2R β expression as the cause of the failure of IL-15 to induce IRF-2−/− BM NK proliferation. In addition, γc subunit expression was equivalent in IRF-2−/− and IRF-2+/+ BM NK cells (data not shown). Is the defective IL-15...
response seen in NK cells restricted only to that cell type? To address this question, we cultured enriched CD8+ T cells in various doses of human recombinant IL-15, and assessed proliferation of CD8+CD44hi T cells by BrdU incorporation using flow cytometry (data not shown). BrdU incorporation was comparable in both IRF-2-/- and IRF-2-/- CD8+ T cells, indicating that no global defect in IL-15 responsiveness exists in IRF-2-deficient mice.

The defect in NK cell differentiation therefore has different underlying causes in IRF-1-/- and IRF-2-deficient mice, suggesting that IRF-1 is not able to compensate for a lack or IRF-2 in this respect.

Discussion

In this study, we present novel data showing that the transcriptional factor IRF-2 is required for Th1 responses and NK cell development. IRF-2-/- mice failed to mount a strong Th1 response to L. major infection, and developed enlarged local skin lesions and highly elevated parasite loads. In contrast to IRF-1-/- mice, which also show increased susceptibility to leishmaniasis (22), IRF-2-/- mice produced normal levels of NO (44), illustrating that the increased susceptibility of IRF-2-/- mice cannot be explained simply by a defect in NOS induction. Rather, IRF-2-/- mice are highly susceptible to L. major infection because of a skewing of their T cell responses towards a Th2 phenotype. A similar bias has been demonstrated in BALB/c mice, the susceptible mouse strain used as the standard for studies in murine experimental leishmaniasis (25).

The Th2 phenotype of IRF-2-/- mice does not reside in a primary defect in the ability of CD4+ T cells to differentiate into Th1 cells. Transfer of purified IRF-2-/-CD4+ T cells into susceptible RAG-1-/- mice was as effective as transfer of IRF-2-/-CD4+ T cells in protecting the recipients against leishmaniasis, and IRF-2-/-CD4+ T cells were fully able to differentiate into Th1 cells in the IRF-2 competent environment of a RAG-1-/- mouse. IRF-2-/-CD4+ T cells were also capable of differentiating into either Th1 or Th2 cells in vitro when cocultured with IL-12 or IL-4, respectively. These data clearly show that, under the appropriate conditions, IRF-2-/-CD4+ T cells are perfectly able to differentiate into Th1 cells and that the defect underlying the Th2 bias of IRF-2-/- mice must encompass cells other than CD4+ T cells.

In fact, our results suggest that defects in several cell types cooperate to create the Th2 phenotype in IRF-2-/- mice. IRF-2-/- macrophages produce reduced amounts of IL-12 after in vitro stimulation (25; and this study), and IL-12p40 mRNA is significantly reduced in the lesion-draining LN s of IRF-2-/- mice. In vivo, macrophages do not produce IL-12 constitutively, but are induced to do so by IFN-γ (45). IFN-γ is a major product of NK cells, and IRF-2-/- mice contain drastically reduced numbers of NK cells. Thus, the defective induction of IL-12 in the absence of early IFN-γ production by NK cells may synergize with the reduced inherent capacity of IRF-2-/- macrophages to produce IL-12 to influence Th1 development. In part, IRF-2-/-CD4+ T cells by themselves may also contribute to the Th2 bias, as indicated by their failure to differentiate in vitro into Th1 cells after anti-CD3 stimulation in the absence of cytokines. Therefore, it is possible that IRF-2 may play a role in a recently described pathway of IL-12-independent Th1 differentiation (46).

A central role for IL-15 in NK cell progenitor differentiation and proliferation has been clearly demonstrated in studies of gene-targeted mice lacking components of the IL-15 receptor (47, 48) or IL-15 itself (49). The lack of NK cells in IRF-1-/- mice has been attributed to a defect in IL-15 production in BM (41, 42). In IRF-2-/- mice, IL-15 transcripts are readily detectable in stimulated IRF-2-/- BM cells and an NK-permissive microenvironment is present because irradiated IRF-2-/- BM chimeras can support wild-type NK cell development. However, IRF-2-/- BM NK cells are immature and show reduced proliferation in response to exogenous IL-15. In contrast to the situation seen in NK cells, IL-15–induced proliferation of CD44hi CD8+ T cells in vitro is comparable in both IRF-2-/- and IRF-2-/- cells, indicating that there is no global defect in IL-15 responsiveness in IRF-2-deficient mice. The precise molecular defect underlying the reduced responsiveness of IRF-2-/- NK progenitors to IL-15 and the developmental retardation of NK cell development is currently under investigation.

In contrast to the NK cell defect, normal numbers of NK+ T cells were present in all IRF-2-/- lymphoid compartments tested. NK+ T cells constitute an interesting IL-4- and IFN-γ-producing subset of T lymphocytes that exhibits a highly skewed TCR repertoire (50). The physiological role of NK+ T cells remains obscure, but may involve the production of IL-4 that occurs immediately after CD3 stimulation (51). Because NK+ T cells are present in normal numbers in IRF-2-/- mice, there may be a relative overabundance of IL-4 (produced by NK+ T cells) compared with IL-12 (produced by NK cell–dependent macrophages) that also biases towards Th2 responses.

We have shown that IRF-2-/- mice are remarkably similar to IRF-1-/- mice (21, 22) in terms of their susceptibility to Leishmania infection, their bias towards a Th2 response, and their cytokine secretion profiles. Furthermore, the macrophages of both IRF-1-/- and IRF-2-/- mice have a diminished capacity to produce IL-12. These results suggest that IRF-2, like IRF-1, mediates at least part of its effects through direct transcriptional activation, possibly via the formation of a complex with another IRF family member. Candidate molecules are IRF-1 itself and IC3BP, which is expressed in cells of the myeloid and lymphoid lineages and which has been shown to interact with IRF-1 (13) and IRF-2 (15). This hypothesis would be favored by the fact that, like IRF-1-/- and IRF-2-/- mice, IC3BP-/- mice display defective Th1 differentiation (24, 25). However, the phenotypes of IRF-1-/- and IRF-2-/- mice differ in three important ways which may at the same time be evidence for in vivo antagonism of IRF-1 by IRF-2, at least for some genes. First, IRF-2-/- mice contain normal numbers of NK+ T cells, whereas these cells are severely...
reduced in IRF-1−/− mice (42). Second, IL-15 expression is enhanced in BM of IRF-2−/− mice, but absent in IRF-1−/− BM. Third, NO synthesis in IRF-1−/− macrophages is almost absent (3) but is normal (44) in IRF-2−/− macrophages. In conclusion, IRF-2 appears to act as an functional agonist rather than antagonist of IRF-1 in the regulation of some, but not all, genes involved in the differentiation of Th cells. In addition, IRF-2 is required for optimal maturation of NK cells.

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