Interferon Regulatory Factor 8 Regulates RANTES Gene Transcription in Cooperation with Interferon Regulatory Factor-1, NF-κB, and PU.1*

Jianguo Liu and Xiaojing Ma

From the Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, New York 10021

Interferon regulatory factor (IRF)-8 is a member of the IRF family of transcription factors important in interferon-γ-mediated signaling and in the development and function of dendritic cells. Regulated on activation, normal T cell expressed and secreted (RANTES, or CCL5) is a member of the CC chemokine family of proteins, strongly chemoattractant for several important immune cell types in host defense against infectious agents and cancer. Here we report that RANTES expression in IRF-8-null macrophages stimulated with interferon-γ and lipopolysaccharide is markedly decreased. IRF-8 can activate RANTES gene transcription in synergism with IRF-1. Interestingly, IRF-8 can activate RANTES transcription independently of IRF-1 through direct physical interactions with NF-κB c-Rel and PU.1 via the NF-κB element located at −88 to −79 in vitro and in vivo. This study uncovers a novel role of IRF-8 in the regulation of RANTES gene expression and the underlying molecular mechanisms whereby IRF-8 interacts with several other important transcription factors to initiate innate immune responses to pathogenic and inflammatory challenges by activating the RANTES gene.

Interferon regulatory factors (IRFs) constitute a family of nine mammalian transcription factors (IRF-1 to -9) that commonly possess a unique helix-turn-helix DNA-binding motif. Members of IRF family are typically induced following microbial infections, and they play important roles in host defense, such as innate and adaptive immune responses and oncogenesis (1).

IRF-8, also known as interferon consensus sequence-binding protein, is an IFN-γ-inducible transcription factor of the IRF family and regulates transcription through multiple target DNA elements, such as IFN-stimulated response element (ISRE), Ets/IRF composite element, and IFN-γ activation site. IRF-8 is restricted in its expression to myeloid and lymphoid cell lineages. It can function both as a transcriptional repressor and an activator, depending on the partners that it interacts with, and it plays crucial roles in myeloid differentiation, generation of plasmacytoid dendritic cells (2), macrophage activation, and tumor suppression (1, 3).

Regulated on activation normal T cell expressed and secreted (RANTES, CCL5) is a member of the CC chemokine family of proteins, which plays an essential role in inflammation by recruiting T cells, macrophages, and eosinophils to inflammatory sites (4–6).

Type I interferons (IFN-α/β) are the key cytokines produced by influenza A virus-infected epithelial cells and monocytes/macrophages (7). IFN-α/β is a major antiviral cytokine, which also has antiproliferative and immunomodulatory functions (8). Influenza A virus-infected monocytes/macrophages secrete MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, MIP-3α, and IP-10 (9), which preferentially favor the recruitment of blood mononuclear cell population to the site of infection (9). Nuclear factor κB (NF-κB), activating protein-1, IRFs, signal transducers and activators of transcription, and nuclear factor-IL-6 (NF-IL-6 or CCAAT/enhancer-binding protein) have been shown to be activated in influenza A virus infection (10–15).

IRF-1, IRF-3, and IRF-7 have been associated with IFN-α/β or IFN-induced gene expression (16). In addition, IRF-9/p48 functions as a DNA-binding component in a IFN-α/β-activated ISGF3 complex that binds to ISREs in the promoters of IFN-α/β-inducible genes. A study by Lin et al. (17) suggests that IRF-3 directly controls RANTES transcription in response to viral infection of human embryonic kidney 293 and Jurkat T cell lines. However, the role of Type II interferon (IFN-γ) in the regulation of RANTES gene transcription was not as well established. An earlier investigation of the transcriptional synergism between IFN-γ and TNFα in activating the RANTES gene in fibroblasts reported cooperation between IFN-γ-activated STAT1α and TNFα-activated NF-κB (18). A subsequent study in the NIH 3T3 fibroblast cell line showed a synergistic activation of RANTES transcription by TNFα and IFN-γ involving direct binding of IRF-1 to a site in the mouse RANTES promoter at −150 to −138, named ISRE (19). In a study of cultured gastric epithelial cells infected with Helicobacter pylori, Kudo et al. (20) showed that maximal H. pylori-induced RANTES gene transcription required the presence of the ISRE, the cyclic AMP-responsive element, NF-IL-6, and two NF-κB sites. Our
own recent study further mapped the IFN-γ-inducible ISRE in the mouse RANTES promoter to −147/−143, which was named IRFI RE (21).

Since IRF-8 is another major IRF member that is induced by IFN-γ and a critical transcription factor for myeloid differentiation and activation in response to microbial infection, we questioned its role in the regulation of RANTES gene expression in macrophages. Thus, we undertook the current study to investigate the role and molecular mechanism of IRF-8 in IFN-γ-mediated induction of RANTES gene transcription in mouse macrophages.

**EXPERIMENTAL PROCEDURES**

**Mice**—Female IRF-8−/− mice and their control littermates (originally obtained from Dr. K. Ozato (National Institutes of Health)) were housed in cages with filter tops in a laminar flow hood and fed food and acid water ad libitum at Weill Medical College of Cornell University Animal Facilities in accordance with the Guide for Laboratory Animal Care by the National Research Council (56). These studies were reviewed and approved by the institutional Animal Care and Use Committee of Weill Medical College.

**Cells**—The murine macrophage cell line RAW 264.7 (hereafter referred to as RAW cells) was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum (Hyclone, Logan, UT) with endotoxin levels of <1 ng/ml. Mouse peritoneal exudate macrophages were obtained by lavage 4 days after injection of sterile 3% thioglycollate broth (1 ml intraperitoneally). Cells were washed and resuspended in RPMI containing 10% fetal calf serum and standard supplements. Macrophages were plated in 24-well tissue culture dishes (1 × 10⁶ cells/well). After a 2-h incubation to allow for adherence of macrophages, monolayers were washed three times to remove nonadherent cells and incubated with RPMI containing 10% fetal calf serum and standard supplements. The next day, IFN-γ (10 ng/ml) and LPS (1 μg/ml) were added at different times.

**Plasmids**—Murine RANTES promoter that extended from −979 to +8 was amplified by PCR with genomic DNA extracted from the spleen of wild type C57BL/6 mice as we previous described (21). Expression vector IRF-8 and control LK440 were previously described (22). All plasmid DNAs for transfection were prepared with Qiagen Endo-free Maxi-Prep kits (Qiagen Inc. Valencia, CA).

**Reagents**—All antibodies used in this study were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Recombinant mouse IFN-γ was purchased from Genzyme (Boston, MA). LPS from Escherichia coli 0217:B8 was purchased from Sigma.

**Semiquantitative RT-PCR**—To determine the level of mouse RANTES mRNA by semiquantitative PCR, cDNA converted from 1 μg of total RNA by gene-specific antisense primer and oligo(dt)₁₂₋₁₈ primer was diluted in several concentrations. Diluted cDNA was mixed with a pair of primers (5 μM) derived from mouse RANTES or GAPDH cDNA sequences and PCR master mix (Invitrogen) in a 25-μl volume. PCR cycling was as follows: 4 min at 94 °C, 30 s at 60 °C, 30 s at 72 °C for 1 cycle followed by 30 cycles at 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The PCR primers used were as follows: forward primer (GGTGGACATAGGGACACACT) and reverse primer (TGGGACGGCAGATCTGAGGG) for mouse RANTES; forward primer (AGAACTCAGCTGCATGAACTG) and reverse primer (CTAGGGAGTTGTGATCCGTCT) for mouse MIG; forward primer (GATCTACGAGTGCTTTTTTG) and reverse primer (GACATACTGGTCGTCTGGAA) for mouse MCP2; forward primer (AAGTTTGGCATTGTGGAAAGG) and reverse primer (AACCATAGGAGGTTGGAACA) for mouse GAPDH.

**Quantitative Real Time PCR**—To determine the level of mRNA of mRANTES by quantitative real time PCR, we used a modified protocol (21). Briefly, cDNA converted from 1 μg of total RNA was diluted in several concentrations. Diluted cDNA was mixed with a pair of primers (10 μM) derived from mouse RANTES or GAPDH cDNA sequences and SYBR green PCR master mix (Applied Biosystems) in a 15-μl volume. PCR cycling was as follows: 2 min at 50 °C, 10 min at 95 °C for 1 cycle followed by 40 cycles at 15 s at 95 °C, 1 min at 60 °C.

**Enzyme-linked Immunosorbent Assays**—Supernatants from murine peritoneal macrophage cultures were harvested at 6, 12, and 24 h after IFN-γ and LPS stimulation and stored at −70 °C. Mouse RANTES protein was detected using the DuoSet enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Concentrations were calculated by regression analysis off the standard curve.

**Nuclear Extract Preparation**—Nuclear extracts for Western blot and electrophoretic mobility shift assay (EMSA) were prepared according to the methods of Schreiber et al. (23).

**Transfection Assay**—Transient transfections were performed by electroporation essentially as previously described (24). Briefly, Murine macrophage cell line RAW264.7 were grown to confluence (10⁶/ml) in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were transfected with mRANTES promoter-reporter constructs. Cells were collected, washed once with RPMI 1640 medium, and resuspended in the same medium at a concentration of 10 × 10⁶ cells/400 µl. 400 µl of cell suspension and 40 µl of DNA (6 μg of RANTES, 6 μg of cytomegalovirus-β-galactosidase, 10 μg of pBlueScriptIIKS+) were placed in 0.45-cm electroporation cuvettes (Gene Pulser; Bio-Rad), and electroporation was carried out at 975 microfarads and 300 V. Transfected cells were collected and resuspended to 1 × 10⁶/ml in RPMI 1640 plus 10% fetal calf serum, and chloroquine was added to a final concentration of 10 μM. Cells were placed in wells (2 ml/well) of a 24-well plate and incubated for 16 h at 37 °C in a 5% CO₂ atmosphere. Cells were treated with recombinant mouse IFN-γ (10 ng/ml) and LPS (1 μg/ml). After a 7-h stimulation, cells were harvested and lysed by Triton. Lysates were used for both luciferase and β-galactosidase assays.

**EMSA**—EMSA and supershifts were performed as described previously (25).

**DNA Affinity Binding Assay**—DNA affinity binding assays were performed essentially as described previously (25). Briefly, complementary biotinylated oligonucleotides encompassing the
murine RANTES IRF-8 response element (IRF-8-RE) site (−99/−67) or the mutant IRF-8-RE were synthesized and annealed. Two micrograms of biotinylated double-stranded DNA were conjugated to 100 μl of streptavidin-bound magnetic beads (Dynabeads, M280; Dynal, Lake Success, NY) in binding/washing buffer for 30 min at room temperature. Conjugated DNA was collected by precipitating by boiling in SDS buffer, fractionated by 12% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Western blotting was performed using a PU.1, IRF-8, or NF-κB c-Rel antibody (Santa Cruz Biotechnologies).

**Chromatin Immunoprecipitation (ChIP) Assay**—The ChIP procedure was performed using an assay kit following the manufacturer’s instructions (Upstate Biotechnology, Inc., Lake Placid, NY) and as previously described (26). The input and immunoprecipitated DNA were amplified by PCR using primers encompassing the IRF-8-RE in the mouse RANTES promoter (5′ primer, GTATTTTGGCCAGAGGGAGGATCTA; 3′ primer, TTATAGGGAGGCCAGGTTACAGACA). The samples were amplified for 30 cycles and analyzed by electrophoresis on a 1.2% agarose gel.

**Statistical Analysis**—Student’s t test was performed where applicable. S.D. is shown unless otherwise indicated (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**RESULTS**

**IRF-8-deficient Macrophages Are Impaired in RANTES Protein, and mRNA Expression Is Induced by IFN-γ and LPS**—To determine the role of IRF-8 in the induction of RANTES protein and mRNA expression, mouse peritoneal macrophages derived from wild type or IRF-8−/− mice were stimulated with LPS alone, IFN-γ alone, and LPS and IFN-γ together, and cell-free culture supernatants were collected and subjected to enzyme-linked immunosorbent assay for RANTES protein secretion, whereas total RNA was isolated and subjected to semi-quantitative RT-PCR analysis. Fig. 1A shows that RANTES protein secretion by IRF-8−/− macrophages stimulated by IFN-γ was almost completely abolished, whereas LPS-stimulated RANTES production was reduced by ~50% over a 24-h period (Fig. 1B). It is noted that LPS is a much more potent
Regulation of RANTES Transcription by IRF-8

FIGURE 3. IRF-8 activates the RANTES promoter in synergy with IRF-1 through IRF1-RE. A, the wild type –979/+8 mouse RANTES promoter-luciferase reporter and the IRF1-RE mutant construct were co-transfected into RAW 264.7 cells with IRF-1 or IRF-8 expression vector or their respective control vector pAct-C (for IRF-1) and LK440 (for IRF-8), and the luciferase activity was measured from total cell lysates. Results are the mean ± S.E. from three independent experiments. B, the same WT and IRF1-RE mutant RANTES promoter constructs were transfected into RAW264.7 cells and stimulated with IFN-γ, LPS, or both for 7 h. Luciferase activity was measured from total cell lysates. Results are the mean ± S.E. from three independent experiments. *p < 0.05, compared with the corresponding conditions between WT and IRF1-RE mutant RANTES promoter constructs.

inducer of RANTES secretion by macrophages than IFN-γ. Adding both IFN-γ and LPS to the cells resulted in RANTES production levels that were partially impaired in IRF-8−/− macrophages at 6 and 12 h poststimulation, whereas by 24 h the level of RANTES secretion was restored to the normal level (Fig. 1C). RANTES mRNA expression in response to IFN-γ and LPS stimulation was impaired in IRF-8−/− macrophages compared with the wild type macrophages by RT-PCR (Fig. 1D) or by real time PCR (Fig. 1E). To determine whether the dependence on IRF-8 was specific to RANTES, we examined MCP2 (a CC chemokine like RANTES) and MIG (a CXC chemokine) in the RT-PCR analysis. Fig. 1D shows that MCP2 expression, but not that of MIG, was also impaired in IRF-8−/− macrophages.

These results indicate that IRF-8 is a critical transcription factor for RANTES gene expression induced primarily by IFN-γ, and to a lesser extent by LPS, in primary mouse macrophages. The discrepancy between equivalent RANTES mRNA expression induced by IFN-γ and LPS on one hand and much higher levels of RANTES protein secretion induced by LPS on the other suggests that LPS may have posttranscriptional effects on RANTES expression.

IRF-8 Increases RANTES Promoter Activation—The above observations suggest that the control of RANTES expression by IFN-γ via IRF-8 is primarily at the level of mRNA synthesis. To investigate further the molecular mechanism whereby IRF-8 regulates RANTES gene transcription in macrophages, transient transfections were carried out in the mouse macrophage cell line RAW264.7 with a 979-bp mouse RANTES promoter (21), together with an IRF-8 expression vector (22). Forced IRF-8 expression in RAW264.7 cells dose-dependently induced the RANTES promoter-driven luciferase reporter activity (Fig. 2A). Activation of RAW264.7 cells with IFN-γ, LPS, or both in the presence of exogenously introduced IRF-8 strongly enhanced RANTES transcription (Fig. 2B).

IRF-8 Can Synergistically Activate the RANTES Promoter with IRF-1—In previously published studies, our group has demonstrated that IRF-8 often acts in synergy with IRF-1 through direct physical interactions to activate the transcription of a number of genes, such as IL-12 p40 and p35 (22, 27). We and others have also identified an
Regulation of RANTES Transcription by IRF-8

IRF-1 response element (IRF1-RE) in the mouse RANTES promoter located at −147 to −143 (19, 21). Therefore, we were interested in determining if IRF-8 could act in synergy with IRF-1 through the IRF-1-RE in the RANTES promoter. Wild type RANTES promoter (−979/+8) and the IRF1-RE mutation construct (21) were co-transfected with IRF-8 and IRF-1 expression vector into the RAW264.7 cells (unstimulated). Fig. 3A shows that the RANTES promoter was synergistically activated by exogenous IRF-8 and IRF-1 in RAW264.7 cells. Moreover, this synergy was dependent on the IRF1-RE, because the IRF-1-RE mutant construct completely lost its ability to respond to IRF-8 and IRF-1 synergistically while retaining the ability to respond to IRF-8 alone (Fig. 3A). The IRF-1-RE is not only important for response to exogenous IRF-1 but also for response to inflammatory stimuli IFN-γ and LPS (Fig. 3B).

To determine if IRF-8 and IRF-1 could bind to the same site, EMSA was performed using a DNA probe spanning the IRF1-RE at −147 to −143 (Fig. 3C). This assay showed that IRF-1 but not IRF-8 could bind to the IRF1-RE in response to IFN-γ but not to LPS (Fig. 3, D and E). These results demonstrate that IRF-8 can synergistically activate RANTES gene transcription with IRF-1 but not through the IRF1-RE.

The NF-κB Element Mediates IRF-8 Response—The observation that the IRF1-RE mutant construct, although having lost its response to IRF-1 alone or to the combination of IRF-1 and IRF-8, could still respond to IRF-8 alone (Fig. 3A) suggested that IRF-8 could independently activate the RANTES promoter via a separate site. To localize this promoter element, designated the IRF-8-RE, we introduced several 5′ deletion constructs of the RANTES promoter from −979 down to −131 and co-transfected these deletion constructs with IRF-8 expression vector in RAW264.7 cells. The response to IRF-8 was similar among all 5′ deletion constructs (data not shown), indicating that the IRF-8-RE must be located downstream of −131.

In search of the IRF-8-RE, we first examined the response of the only established promoter element downstream of −131 of the mouse RANTES promoter (i.e. the NF-κB element at −88/−79) (see sequence in Fig. 4A) (19) to IRF-8 by transfecting into RAW264.7 cells the wild type and NF-κB mutant constructs of the RANTES promoter. The NF-κB mutant construct containing a 3-bp substitution at −81 to −79 was far less responsive to stimulation with LPS, IFN-γ, or both as shown by reduced basal transcription (Fig. 4B). To our surprise, the mutant construct also lost its response to IRF-8 (Fig. 4C), indicating that the NF-κB element is also an IRF-8-RE.

To determine if IRF-8 induces RANTES transcription through the NF-κB element by direct or indirect mechanisms, we performed DNA affinity binding assay using wild type and NF-κB mutant oligonucleotide probes (Fig. 4D). Fig. 4E shows that IRF-8 specifically bound to the NF-κB element only in response to both IFN-γ and LPS and not to either stimulus alone or to the mutant probe.

IRF-8, NF-κB, and PU.1 Bind to the NF-κB Site and Promote RANTES Transcription—To further dissect the physical characteristics of the IRF-8 effect on RANTES transcription via the NF-κB element, EMSA was performed using nuclear extracts isolated from RAW264.7 cells stimulated with IFN-γ, LPS, or IFN-γ plus LPS and the −99/−67 sequence containing the NF-κB site as the probe (Fig. 4D). As shown in Fig. 5A, there were two groups of binding activities formed on the probe. Group I appeared to be inducible by IFN-γ alone slightly (lane 3) but more by LPS (lane 4) or IFN-γ plus LPS (lane 5). Group II binding activities were constitutively present and not affected significantly by the stimuli. Competitive EMSA (Fig. 5B) showed that Group I binding could be efficiently competed off by unlabeled probe itself (lane 7) but not by NF-κB mutant (lane 8). A “supershift” experiment using specific antibodies to various members of the NF-κB family (Fig. 5C) demonstrated that this complex contains predominantly NF-κB p50 (lane 11).
Regulation of RANTES Transcription by IRF-8

FIGURE 5. IRF-8, PU.1, and NF-κB bind to RANTES promoter in vitro. A, nuclear extracts were isolated from RAW264.7 cells following IFN-γ or LPS stimulation or combination of both for 5 h. EMSA was performed with 10 μg of nuclear extract for each sample and a double-stranded oligonucleotide probe containing the −99/−67 region of the mouse RANTES promoter. There were two groups of binding activities formed on the probe. Group I appeared to be inducible by IFN-γ alone slightly but more by LPS or IFN-γ plus LPS. Group II binding activities were constitutively present and not affected significantly by the stimuli. B, competition EMSA was performed with the −99/−67 probe and a mutant NF-κB probe (see Fig. 4D) as competitor as indicated. A supershift EMSA was performed with the −99/−67 probe and nuclear extracts from either LPS-stimulated (C) or IFN-γ-treated cells (D). A series of NF-κB-related (C) or PU.1-related (D) antibodies and their control rabbit IgG were used. E, RAW264.7 cells were transiently co-transfected with WT or PU.1 mutant RANTES promoter together with the IRF-8 expression vector or its control vector as indicated. Cells were not further stimulated before luciferase activity measurement. Data represent the mean ± S.E. from three independent experiments. F, RAW264.7 cells were transiently transfected with WT RANTES promoter together with the IRF-8 expression vector and PU.1 dominant negative mutant (DNM) or their control vector LK440 or CB6, respectively. Relative luciferase activity was calculated as -fold level of expression vectors versus its control vectors under the medium condition. Data represent the mean ± S.E. from three independent experiments.

and c-Rel (lane 13) and to a lesser extent p65 (lane 12) and not p52 (lane 14).

Group II binding appeared similar to PU.1 binding to the human IL-12 p40 promoter at a GGAA motif immediately upstream of the NF-κB site that we previously studied (28). Indeed, there is a GGAA sequence at the 5′-end of the NF-κB element (−88/−85) (Fig. 4D). A PU.1-specific antibody strongly retarded/diminished these binding activities (lane 16; Fig. 4D) but not by antibodies directed toward two other members of the PU.1 family, Est1 and Est2 (lanes 17–19).

Previous studies have demonstrated that IRF-8 can interact with other transcription factors, including PU.1, to regulate the transcription of target genes (29). To determine the functional relationship between IRF-8 and PU.1 with respect to their role in regulating RANTES transcription, we tested the effect of the PU.1 site mutation on the RANTES promoter response to IRF-8. As shown in Fig. 5E, base substitutions at the PU.1 motif within the NF-κB element totally inhibited the promoter activity in response to IRF-8. Furthermore, we co-transfected IRF-8 and a PU.1 dominant negative mutant (30) with the RANTES promoter-reporter. The PU.1 dominant negative mutant (DNM) completely abolished the effect of IRF-8 (Fig. 5F). Together with the data in Fig. 4, these results strongly suggest that IRF-8, NF-κB, and PU.1 functionally interact with the NF-κB element to promote RANTES transcription.

IRF-8 Directly Interacts with PU.1 and NF-κB in Vitro and in Vivo—The functional interdependence between IRF-8, NF-κB, and PU.1 led us to question if they could directly interact with each other physically. An immunoprecipitation assay was performed in IFN-γ-treated RAW264.7 cells using the anti-IRF-8 antibody, followed by Western blot with anti-PU.1, IRF-8, and c-Rel antibodies. Clearly, IFN-γ induced direct physical association of these three factors (Fig. 6A, lane 4). It is noteworthy that although PU.1 is constitutively expressed (lane 5), its interaction with IRF-8 and c-Rel depends on IFN-γ stimulation.

To determine whether IRF-8 could bind to the endogenous RANTES promoter around the NF-κB site in vivo in primary mouse macrophages, we performed ChIP using a pair of primers flanking the RANTES promoter-reporter. The PU.1 dominant negative mutant (DNM) completely abolished the effect of IRF-8 (Fig. 5F). Together with the data in Fig. 4, these results strongly suggest that IRF-8, NF-κB, and PU.1 functionally interact with the NF-κB element to promote RANTES transcription.

DISCUSSION

IFN-γ is a pivotal effector cytokine in immune responses. It is produced during the innate phase of immunity by NK cells in response to viral infection. It is produced by activated CD4+ and CD8+ T cells during the adaptive phase of immunity to drive cell-mediated immunity against intracellular pathogens (31, 32). IFN-γ is also essential in immunosurveillance against malignant transformation (33).

The critical importance of IRF-8 for IFN-γ signaling
Regulation of RANTES Transcription by IRF-8

FIGURE 6. IRF-8 associates with NF-κB and PU.1 in vitro and binds to endogenous RANTES promoter in vivo. A, co-immunoprecipitation was performed with nuclear extract isolated from resting or IFN-γ-stimulated RAW264.7 cells with the anti-IRF-8 antibody (top), anti-NF-κB antibody (middle), and anti-c-Rel antibody (bottom). B, sequence of the mouse RANTES promoter region (−231 to −12) containing IRF1-RE, NF-κB, and PU.1 elements. The sequences of the pair of PCR primers used to perform ChIP are underlined. C, ChIP analysis was performed in WT mouse peritoneal macrophages. The amplified mouse genomic fragment derived from the endogenous RANTES promoter encompassing the above-described elements is indicated. The control antibody was an isotype-matched IgG. Input DNAs were used as loading controls.

implies its essential role in regulating immune responses. Mice deficient in IRF-8 manifest a syndrome similar to human chronic myelogenous leukemia (CML). Approximately 33% of IRF-8−/− and 9% of IRF-8+/− became moribund (blast crisis) by 50 weeks of age (34). Many types of hematopoietic neoplasms develop in AKXD recombinant inbred, NFS.1+, and IRF-8 knock-out mice that relate to human lymphoma and leukemia (35). The majority of human CML patients have a strong deficiency in IRF-8 mRNA expression (36). High IRF-8 levels were only observed in “good responders” to IFN-α treatment in CML patients (37). Expression of IRF-8 is down-regulated in BCR/Ab1-induced murine CML-like disease, and enforced co-expression of IRF-8 protein attenuates and alters the disease phenotype induced by Bcr-Ab1 (38). However, the mechanism of IRF-8 and/or whether some other immune factors play any roles in the development of CML is unclear.

Our previous study demonstrated that IRF-1 is required for RANTES expression by macrophages in response to IFN-γ (21). The current findings show that IRF-8, like IRF-1, is required for IFN-γ-induced RANTES expression (Fig. 1A). However, unlike IRF-1, IRF-8 is also partially required for LPS-induced RANTES expression (Fig. 1B). This differential requirement is probably due to the unique ability of IRF-8 to interact with IRF-1 in the IFN-γ response through the IRF1-RE/ISRE at −147/−143 of the RANTES promoter and to interact directly with NF-κB and PU.1 at the NF-κB site at −88/−79 in the IFN-γ and LPS responses (Figs. 4 and 5). The ability of IRF-8 to interact with PU.1 in response to IFN-γ activation has been demonstrated previously on IFN-γ-induced p67phox and gp91phox expression (39), IL-1β (40), Disabled-2 (41), IFN-stimulated gene 15 (42), and p15ink4b (43). Moreover, using a reporter assay system employing a self-inactivating retrovirus, Tamura et al. (44) identified several IRF-8 target genes with a consensus sequence GAAANN(N)GGAA (where (N) means any nucleotide, either present or absent) comprising a core IRF-binding motif and an Ets-binding motif; this sequence is similar but distinct from the previously reported Ets/IRF composite element. ChIP assays demonstrated that IRF-8 and the PU.1 bind to this element in vivo (44). PU.1 belongs to the Ets family of DNA-binding proteins (45, 46). It is expressed predominantly in macrophages, B cells, and erythroid cells (47, 48). PU.1 plays important but varied roles in the development of hematopoietic cells. A deletion of the PU.1 gene leads to a failure to produce mature B lymphocytes and macrophages (49). In many instances, the transcriptional activation by PU.1 depends on the cooperation of PU.1 with other transcription factors. These factors can either become physically associated with PU.1 or bind to DNA adjacent to the Ets site upon which PU.1 resides (29, 50–53).

The independent ability of IFN-γ and LPS to activate the RANTES promoter on the one hand and the lack of synergy between the two stimuli on the promoter activity on the other (Fig. 4B) are seemingly inconsistent with the observation that synergistic IRF-8 binding to the NF-κB site was induced only by a combination of both stimuli (Fig. 4E). Our interpretation is the following: 1) IFN-γ alone induces RANTES transcription through activation of IRF1 and IRF-8, which synergistically bind to the IRF1-RE (Fig. 3, A and B); 2) LPS alone activates RANTES transcription primarily through induction of NF-κB (Figs. 4B and 5A); 3) although IRF-8 expression is moderately inducible by LPS and highly inducible by IFN-γ, IRF-8 cannot bind to the NF-κB site unless both stimuli are present (Fig. 4E); and 4) the synergistic binding of IRF-8 to the NF-κB site induced by both IFN-γ and LPS does not cause a synergistic activation of the RANTES transcription.

It is consistent and interesting that IRF-8 could directly interact with NF-κB c-Rel (Fig. 6A). Tsujimura et al. (54) reported that dendritic cells from IRF-8−/− mice were unresponsive to CpG (a TLR9 ligand) and failed to induce TNF-α and IL-6, targets of NF-κB, whereas these cytokines were robustly induced in response to LPS (a TLR4 ligand). Further analysis revealed that CpG failed to activate NF-κB in IRF-8−/− cells. This was due to the selective inability of IRF-8−/− dendritic cells to activate IκB kinase αβ, which are required for NF-κB in response to CpG (54). Our unique finding suggests that IFN-γ induces direct physical interaction between IRF-8 and c-Rel in macrophages. However, the impact of this interaction on the transcription of the RANTES gene induced by the combination of IFN-γ and LPS is not on the synergism; rather, it is a required component of the transcriptional complex that forms on this site in response to LPS, the absence of which would result in diminished RANTES transcription (Fig. 1B).

Taken together, the phenotype of CML-like syndrome in the
IRF-8−/− animals can have an immunological basis. First, IFN-γ plays an essential role in cancer immunosurveillance, and it depends critically on IRF-1 and IRF-8 for its immunological activities (e.g. for IL-12 production (27) and RANTES expression (21), which are important regulators of NK/T cell activation and leukocyte migration, respectively). Second, IRF-8 has additional vital roles in myeloid differentiation, generation of plasmacytoid dendritic cells (2), macrophage activation, and tumor suppression (55).

In conclusion, our data have unequivocally established that IRF-8 is an essential transcription mediator for IFN-γ- and LPS-induced RANTES gene expression in macrophages. IRF-8 can physically interact with IRF-1, c-Rel, and PU.1, upon inflammatory or pathogenic challenges, to activate the RANTES gene in a synergistic manner through two separate promoter elements, the ISRE/IRF1-RE at −147/−143 and the NF-κB site at −88/−79, respectively. The defining of a novel pathway of RANTES induction via IRF-8 will probably contribute to a better understanding of chemokine-mediated immune cell migration in response to inflammatory/pathogenic insults.

REFERENCES
1. Taniguchi, T., Ogasa, K., Takaoka, A., and Tanaka, N. (2001) Annu. Rev. Immunol. 19, 623–655
2. Tsujimura, H., Tamura, T., and Ozato, K. (2003) J. Immunol. 170, 1131–1135
3. Tamura, T., Kong, H. J., Yu, Y. C., Tsujimura, H., Calame, K., and Ozato, K. (2003) Blood 102, 4574–4554
4. Schall, T. J., Brunner, T., Bischoff, S. C., Schall, T. J., and Dahinden, C. A. (1992) J. Exp. Med. 167, 1149–1495
5. Meurer, R., Van Riper, G., Feeney, W., Cunningham, P., Hora, D., Jr., Springer, M. S., MacIntyre, D. E., and Rosen, H. (1993) J. Exp. Med. 178, 1913–1921
6. Ronn, T., Sareneva, T., Pirinen, J., and Julkunen, I. (1995) J. Immunol. 154, 2764–2774
7. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 227–264
8. Sprenger, H., Meyer, R. G., Kaufmann, A., Bussfeld, D., Rischkowsky, E., and Gems, D. (1996) J. Exp. Med. 184, 1191–1196
9. Mattikainen, S., Pirinen, J., Miettinen, A. M., Lehtonen, A., Govenius-Vintola, C., Sareneva, T., and Julkunen, I. (2000) Viral. Cell. 62, 138–147
10. Ronn, T., Mattikainen, S., Sareneva, T., Melen, K., Pirinen, J., Keskinen, P., and Julkunen, I. (1997) J. Immunol. 158, 2363–2374
11. Choi, A. M., Knohl, K., Otterbein, S. L., Eastman, D. A., and Jacoby, D. B. (1996) Ann. J. Physiol. 271, L383–L391
12. Hofmann, P., Sprenger, H., Kaufmann, A., Binder, A., Hasse, C., Nairn, M., and Gems, D. (1997) J. Leukoc. Biol. 61, 408–414
13. Pahl, H. L., and Baueerle, P. A. (1995) J. Virol. 69, 1480–1484
14. Flory, E., Kunz, M., Scheller, C., Jassey, C., Stauber, R., Rupp, U. R., and Ludwig, S. (2000) J. Bacteriol. 275, 8307–8314
15. Mammone, Y., Heybrock, C., Genin, P., Algarte, M., Servant, M. J., LePage, C., Deluca, C., Kwon, H., Lin, R., and Hiscott, J. (1999) Gene (Arst.) 237, 1–14
16. Lin, R., Heybrock, C., Genin, P., Pitka, P. M., and Hiscott, J. (1999) Mol. Cell. Biol. 19, 959–966
17. Ohmori, T., Schreiber, R. D., and Hamilton, T. A. (1997) J. Biol. Chem. 272, 14899–14907
18. Lee, A. H., Hong, J. H., and Seo, Y. S. (2000) Biochem. J. 350, 131–138
19. Kudo, T., Li, H., Wu, Y. J., Graham, D. Y., Casola, A., and Yamaoka, Y. (2005) Infect. Immun. 73, 7602–7612
20. Liu, J., Guan, X., and Ma, X. (2005) J. Biol. Chem. 280, 24347–24355
21. Wang, L., Contursi, C., Masumi, A., Ma, X., Trinchieri, G., and Ozato, K. (2000) J. Immunol. 165, 271–279
22. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
23. Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. F., Dzialo, R., and Trinchieri, G. (1996) J. Exp. Med. 183, 147–157
24. Ma, X., Neurath, M., Gri, G., and Trinchieri, G. (1997) J. Biol. Chem. 272, 10389–10395
25. Liu, J., Cao, S., Herman, L. M., and Ma, X. (2003) J. Exp. Med. 198, 1265–1276
26. Liu, J., Guan, X., Tamura, T., Ozato, K., and Ma, X. (2004) J. Biol. Chem. 279, 55609–55617
27. Gri, G., Savio, D., Trinchieri, G., and Ma, X. (1998) J. Biol. Chem. 273, 6431–6438
28. Eklund, E. A., Jalava, A., and Kakar, R. (1998) J. Biol. Chem. 273, 13957–13965
29. Grazia Cappiello, M., Sutterwala, F. S., Trinchieri, G., Mosser, D. M., and Ma, X. (2004) J. Immunol. 172, 6820–6827
30. Tailor, P., Tamura, T., and Ozato, K. (2006) Cell Res. 16, 134–140
31. National Research Council (1985) Guide for the Care and Use of Laboratory Animals, Publication 85-23, National Institutes of Health, Bethesda, MD

JULY 14, 2006 • VOLUME 281 • NUMBER 28

JOURNAL OF BIOLOGICAL CHEMISTRY

19195