Interferon Regulatory Factor 1 Is an Essential and Direct Transcriptional Activator for Interferon γ-induced RANTES/CCL5 Expression in Macrophages*

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Interferon regulatory factor 1 (IRF-1) is an important transcription factor in interferon γ (IFNγ)-mediated signaling in the development and function of NK cells and cytotoxic T lymphocytes. RANTES (regulated on activation normal T cell expressed and secreted; CCL5) is a member of the CC chemokine family of proteins, which is strongly chemoattractant for several important immune cell types in host defense against infectious agents and cancer. However, the role of IFNγ and IRF-1 in the regulation of RANTES gene expression and their operative mechanisms in macrophages have not been established. We report here that RANTES expression in IRF-1-null mice, primarily in macrophages, in response to carcinogenic stimulation in vivo and in vitro and to IFNγ but not to lipopolysaccharide in vitro, was markedly decreased. As a result, RANTES-mediated chemoattraction of CCR5+ target cells was also severely impaired. Adenovirus-mediated gene transduction of IRF-1 in primary macrophages resulted in enhanced RANTES expression. The IFNγ and IRF1 response element was localized to a TTTTC motif at −147 to −143 of the mouse RANTES promoter, to which endogenous or recombinant IRF-1 can physically bind in vitro and in vivo. This study uncovers a novel IFNγ-induced pathway in RAN-
TES expression mediated by IRF-1 in macrophages and elucidates an important host defense mechanism against neoplastic transformation.

Interferon regulatory factors (IRFs)1 constitute a family of nine mammalian transcription factors (IRF-1 to -9) that commonly possess a unique helix-turn-helix DNA-binding motif. The first discovered member of this family, IRF-1, has a remarkable functional diversity in the regulation of cellular responses in host defense. IRF-1 targets different sets of genes in various cell types in response to diverse cellular stimuli and evokes appropriate innate and adaptive immune responses (1). It has been firmly established as a critical effector molecule in IFNγ-mediated signaling and in the development and function of NK cells, NK T cells, and cytotoxic T lymphocytes (2–7). IRF-1 also has direct antiproliferative effects, thus acting as a tumor suppressor and tumor susceptibility gene (8).

In a recently published study aimed at elucidating the role of IRF-1 in immune surveillance against T cell lymphoma development, we reported that IRF-1-deficient mice were highly susceptible to N-methyl-N-nitrosourea (MNU)-induced T lymphomas (9). By DNA microarray analysis, we comprehensively identified differences and patterns in gene expression in splenocytes of wild type (WT) versus IRF-1−/− mice challenged with MNU. IRF-1 mRNA expression was induced by MNU in vivo. One of the interesting genes differentially affected in MNU-induced lymphoma was RANTES (regulated on activation normal T cell expressed and secreted; CCL5). RANTES is a member of the CC chemokine family of proteins that plays an essential role in inflammation by recruiting T cells, macrophages, and eosinophils to inflammatory sites (10–12). RAN-
TES expression is also a predictor of survival in Stage I lung adenocarcinoma (13). Coexpression of RANTES and B7.1 have been shown to elicit strong antitumor and recall responses as well as tumor-specific cytotoxic T cell activity when delivered intratumorally with a herpes simplex virus ampiclon vector in a murine model of preestablished EL4 T lymphoma (14). Our microarray data strongly indicate that IRF-1 mediates MNU-induction of RANTES expression in vivo (9). However, the mechanism by which IFNγ and IRF-1 regulate RANTES gene expression in primary macrophages has been rarely explored. A study by Lin et al. (15) suggests that IRF-3 directly controls RANTES transcription in response to viral infection of human embryonic kidney 293 and Jurkat T cell lines. An earlier investigation of the transcriptional synergism between IFNγ and TNFα in activating the RANTES gene in fibroblasts reported cooperation between IFNγ-activated STAT1α and TNFα-activated NFκB (16). A subsequent study in NIH 3T3 fibroblast cell line showed a synergistic activation of RANTES transcription by TNFα and IFNγ involving direct binding of IRF-1 to a site in the mouse RANTES promoter at −150 to −138 (17). Moreover, one study showed that IFNγ induced RANTES gene expression by stabilizing RANTES mRNA in alveolar epithelial cells (18). Thus, we undertook the current study to investigate the role and molecular mechanism of IRF-1 and IFNγ in direct regulation of RANTES gene transcription in mouse macrophages using IRF-1−/− mice.

MATERIALS AND METHODS

Mice—Female IRF-1−/− mice and their control, C57BL/6J mice (6–8 weeks old), were obtained from The Jackson Laboratory (Bar Harbor, Maine).
ME). All of the mice 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 National Institutes of Health guidelines on the care and use of the animals.

**Cells**—The murine macrophage cell line RAW264.7 (RAW cells hereafter) was obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin and streptomycin, and 10% fetal bovine serum (HyClone, Logan, UT; endotoxin < 1 ng/ml). U77 cells stably transfected with human CCR5 were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin and streptomycin. Mouse peritoneal exudate macrophages were obtained by lavage 4 days after the injection of sterile 3% thioglycollate broth (1 ml intraperitoneally). Cells were washed and resuspended in RPMI 1640 medium containing 10% fetal calf serum and standard supplements. Macrophages were plated in 24-well tissue culture plates (1·10⁶ cells/well). After a 2-h incubation to allow for the adherence of macrophages, monolayers were washed three times to remove nonadherent cells and incubated with RPMI 1640 medium containing 10% fetal calf serum and standard supplements. The next day IFNγ (10 ng/ml) and LPS (1 µg/ml) were added at different time points. The U77,CD4.CCR5 cell line (human glioma cell line) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health, from Drs. HongKui Deng and Dan Littman.

**Plasmids**—Murine RANTES promoter that extended from −979 to +8 was amplified by PCR with genomic DNA extracted from wild-type C57BL/6 mice (5′-primer, ATCTCTTGTGGCCC ACCA; 3′-primer, TG-CAGGGGGTGGCTGTCGA). The PCR product was cloned into PCR2.1 cloning vector. After sequence verification the insert in PCR2.1 was cut out with XhoI and Hind III and cloned into pGL2-basic luciferase vector. IRF-1 mutants (M1 to M3) were generated by site-directed mutagenesis according to the manufacturer’s protocol (Strategene, Kingsport, TN). Expression vectors pAct-1 (IRF-1), pAct-2 (IRF-2), and control pAct-C were generously provided by Dr. T. Taniguchi (University of Tokyo, Japan). The expression vectors for NFκB p50, p65, and c-Rel were provided by Dr. K. Murphy (University of Washington, St. Louis, MO) and have been described previously (19). All plasmid DNA for transfection was prepared with Endo-free Maxi-Prep kits (Qiagen Inc., Valencia, CA).

**Reagents**—Antibodies for IRFs used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant mouse RANTES protein and RANTES antibody were obtained from R&D System Inc. (Minneapolis, MN). Recombinant mouse IFNγ was purchased from Genzyme (Boston, MA). LPS from Escherichia coli 0217:BS was purchased from Sigma-Aldrich.

**RNase Protection Assay**—Mouse macrophages were pretreated with IFNγ for 16 h followed by treatment with LPS for an additional 4 h. Ten µg of total RNA for each condition was subject to analysis by multiprobe RNase protection assay using the MC-5c probe set (BD Biosciences) according to the manufacturer’s instructions.

**Reverse Transcription-PCR**—RT-PCR reactions were carried out under standard conditions. The following primers were used for PCR amplification of the mouse RANTES cDNA: sense primer, 5′-GATGGG-ACTAGGACGACAAC-3′, and antisense primer, 5′-TGGAGCGCA-GATCTGAGGG-3′; mouse hypoxanucleo-guanine phosphoribosyltransferase sense primer, 5′-TTGGTATACAGGCCAGACTTTGTTG-3′, and antisense primer, 5′-GAGGAGTCGGCTCCTATGGCT-3′.

**Quantitative Real-time PCR**—To determine the level of RANTES mRNA and IRF-1 mRNA in noninfected mouse peritoneal macrophages in vivo, we used a modified protocol described by Rajeevan et al. (20). 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 and 10 min at 95 °C for 1 cycle followed by 40 cycles at 15 s at 95 °C and 1 min at 60 °C. The PCR primers used were: forward primer, 5′-GATGGCAGTATAGGACACAAC-3′, and reverse primer, 5′-TGGAGCGCA-GATCTGAGGG-3′; mouse RANTES; forward primer, 5′-GAGGAGTCGGCTCCTATGGCT-3′, and reverse primer, 5′-AACATTTGGGTGGTAGACCA-3′, for mouse GAPDH.

**Enzyme-linked Immunosorbent Assays**—Sera from noninfected peritoneal macrophage cultures were harvested at 6, 12, and 24 h after IFNγ and LPS stimulation and stored at −70 °C. Mouse RANTES was detected by using the DuoSet ELISA kits (R&D Systems) according to the manufacturer’s instructions. Concentrations were calculated by regression analysis of a standard curve.

**Chemotaxis Assay**—A cell migration assay was performed in a 96-well microchemotaxis double-chamber (NeuroProbe, Gaithersburg, MD) using U77.CD4 cells stably transfected with CCR5, which would migrate toward a RANTES gradient (21) as follows. 29 µl of supernatant from WT and IRF-1−/− macrophages treated with IFNγ or LPS was plated into the lower chamber. Different amounts of recombinant mouse RANTES (0.25–10 µg/ml), serving as positive controls, were added to the wells in the lower chamber in complete RPMI 1640 medium containing 10% fetal calf serum. As a negative control, the same volume of complete RPMI 1640 medium was used. After separation by polynivinylpyrrolidone-free polycarbonate filters with 5-µm pores (NeuroProbe), the upper chamber was filled with 50 µl of U77-CCR5+ cell suspension (2×10⁶ cells/ml). The chamber was incubated at 37 °C in an incubator with 5% CO₂ for 3.5 h. Thereafter, filters with migrated U77-CCR5 cells were removed, fixed, stained by the Hema3 system (Fisher, Middleton, VA), and counted. The total number of migrated cells per filter was densitometrically calculated with an Olympus microscope (Olympus Optical, Tokyo, Japan) equipped with a Spot digital camera (Sony, Tokyo, Japan).

**Nuclear Extract Preparation**—Nuclear extracts for Western blot and electrophoretic mobility shift assays (EMSA) were prepared according to the methods of Schreiber et al. (22).

**Adenoviral Vectors and Their Propagation**—The construction and propagation of LacZ- and IRF-1-expressing adenoviruses were described elsewhere (19).

**Transfection Assay and EMSA**—Transient transfections were performed by electroporation as described previously (23). EMSA and supershifts were performed as described previously (24).

**Chromatin Immunoprecipitation (ChIP) Assay**—The ChIP procedure was performed using an assay kit following the manufacturer’s instructions (Upstate Biotechnology, Lake Placid, NY) as described previously (19). The input and immunoprecipitated DNA were amplified by PCR using primers encompassing the IRF1-RE in the mouse RANTES promoter (5′-primer: GTATTGGCCAGAGGGAGTCTCAT; and 3′-primer: TTTATAGGAGCCAGGTTAGCAGA). 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 wherever applicable. S.D. of the mean is shown unless otherwise indicated.

**RESULTS**

**IRF-1-deficient Mouse Have Impaired Expression of RANTES Protein and mRNA Expression in Response to Carcinogen**—In the original study in which we first characterized the gene expression changes in mice challenged with the lymphoma-inducing carcinogen MNU, RANTES mRNA expression was noted to be severely impaired in IRF-1−/− mice (9). However, the analysis was performed with the spleen of the mice 4 months after the MNU treatment when the architecture of the organ in IRF-1−/− mice suffering severe lymphoma had been drastically altered compared with the more resistant wild type control mice, thus raising concerns about the validity of the comparison. To alleviate the concern we reperformed the analysis using control and IRF-1−/− mice 1 week after the MNU treatment when the spleen composition of the mice was still largely intact. As shown in Fig. 1A, RANTES protein level in the serum was detectable in nontreated wild type but not in IRF-1−/− mice. One week following the single injection of MNU, elevated RANTES level was observed in the serum of WT mice but not in IRF-1−/− mice. Quantitative real-time PCR analysis of mRNA expression in the spleen of these mice showed a similar phenotype in that IRF-1−/− mice had strongly reduced RANTES transcripts with or without MNU challenge (Fig. 1B). Consistent with RANTES mRNA expression, quantitative RT-PCR also showed that IRF-1 was induced by MNU in WT mice (Fig. 1C). These results confirmed our previous finding at both protein and mRNA levels and suggested that both the basal and the carcinogen-induced RANTES expression is largely dependent on IRF-1 in vivo. Within the spleen, adherent cells (macrophages) appeared to be much more potent producers of RANTES than did nonadherent cells (lymphocytes) directly in response to stimulation on a per cell basis given that lympho-
cytes by far outnumber macrophages in the spleen, because the adherent cells showed a dose response, whereas lymphocytes did not respond to MNU stimulation (Fig. 1D).

**IRF-1-deficient Macrophages Have Impaired Expression of RANTES mRNA and Protein Induced by IFNγ but Not by LPS—**To determine the role of IRF-1 and IFNγ in the induction of RANTES mRNA expression, we isolated RNA from peritoneal macrophages derived from wild type or IRF-1−/− mice following in vitro stimulation with LPS, or IFNγ, or both. The RNA samples were subjected to RNase protection assay (Fig. 2A) and RT-PCR (Fig. 2B), and the cell culture supernatants were subjected to ELISA for RANTES protein secretion (Fig. 2, D–F). Fig. 2A shows that although the wild type macrophages expressed RANTES mRNA in response to IFNγ stimulation (lane 2, top band), IRF-1−/− macrophages were nonresponsive to IFNγ (lane 6). On the other hand, both types of cells were equally responsive to LPS stimulation (Fig. 2A, lanes 3 and 7). The combination of IFNγ- and LPS-induced RANTES mRNA expression reflected the deficit in IFNγ response in IRF-1−/− mice (compare lanes 4 and 8 in Fig. 2A). This deficiency was persistent because extended stimulation with IFNγ could not alter the lack of response (Fig. 2B). These data were further confirmed by quantitative RT-PCR in that IFNγ-induced RANTES mRNA expression was deficient in IRF-1−/− macrophages (Fig. 2C). The selective impairment of RANTES expression was strongly echoed at the protein level, as RANTES protein secretion by IRF-1−/− macrophages stimulated by IFNγ (Fig. 2D) were severely abrogated but not those stimulated by LPS (Fig. 2E) or by a combination of IFNγ and LPS (Fig. 2F).

Note that LPS is a much more potent inducer of RANTES expression in macrophages than is IFNγ (different scales are used in Fig. 2D and Fig. 2, E and F). These results suggest that IRF-1 is an essential transcription factor for RANTES gene expression induced by IFNγ in mouse primary macrophages.

**Migration of CCR5+ Cells toward IFNγ-stimulated IRF-1−/− Macrophages Is Impaired—**The above results indicate that IRF-1 is critical for RANTES gene expression. We were interested in determining whether the deficiency in RANTES production in IRF-1−/− macrophages would translate into the impairment in RANTES-mediated migration. We performed cell migration experiments using U87.CD4 cells (human glioma cell line) stably transfected with CCR5, which would migrate toward a RANTES gradient, among others macrophage inflammatory protein-1α and -1β (21), in a double-chambered ChemoTx system (NeuroProbe). Fig. 3A shows that the CCR5+ U87.CD4 cells responded to recombinant RANTES-mediated chemotactic signal in a dose-dependent manner. This chemotaxis was observed also with IFNγ- or LPS-activated murine macrophages as a source of chemokines in a RANTES-dependent manner because neutralization of RANTES using a monoclonal antibody largely blocked the migration of the CCR5+ cells (Fig. 3B). Moreover, IRF-1−/− macrophages stimulated with IFNγ, but not with LPS, were strongly deficient in inducing CCR5+ chemotaxis (Fig. 3C). These results clearly demonstrate that IRF-1 is a critical regulator of RANTES expression and RANTES-mediated cell migration.

**IRF-1 Expression Increases Mouse RANTES Production in Primary Macrophages andInduces RANTES Promoter Activation—**To confirm the inductive role of IRF-1 on RANTES gene expression in primary macrophages, we carried out a gene...
delivery experiment using adenovirus expressing IRF-1. As shown in Fig. 4A, the IRF-1-adenovirus-infected HEK293 cells expressed very high levels of IRF-1 protein both in the cytoplasm and in the nucleus 48 or 72 h postinfection, whereas the control virus expressing the LacZ gene did not show such expression of IRF-1 protein. Furthermore, the IRF-1 adenovirus-transduced mouse peritoneal macrophages displayed increased RANTES protein production in a dose-dependent manner in correlation with increased IRF-1 expression (Fig. 4B). Again, these data strongly support the notion that IRF-1 is a critical transcriptional activator for RANTES expression.

The results shown in Fig. 2 suggest that the control of RANTES expression by IFNγ via IRF-1 is primarily at the level of mRNA synthesis. To investigate further the molecular mechanism whereby IRF-1 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, cloned by PCR exactly as described by Lee et al. (17) or together with an IRF-1- or IRF-2-expression vector. The RANTES promoter-driven luciferase activity was greatly induced by either IFNγ or LPS and additively by both (Fig. 4C). IRF-1 expression in unstimulated RAW cells dose-dependently induced the RANTES promoter activity with a peak at 0.75 μg of the IRF-1-expression vector, whereas additional increases in IRF-1 reduced its transactivation potency (Fig. 4D). In contrast, IRF-2 expression dose-dependently inhibited the RANTES promoter activity (Fig. 4E). The endogenous IRF-1 and IRF-2 were present in an IFNγ-inducible (IRF-1) or -constitutive (IRF-2) manner (Fig. 4F). These results demonstrate that IFNγ can induce RANTES gene transcription and that overexpression of IRF-1 can activate transcription of the RANTES gene, whereas IRF-2 may be a transcriptional inhibitor.

Localization of the IRF-1 Response Element in RANTES Promoter—To further confirm the role of IFNγ/IRF-1 in the transcriptional regulation of RANTES we introduced base substitutions into the RANTES promoter (Fig. 5A) and compared their activity in the context of the full-length RANTES promoter (−979 to +8). Three mutant constructs were made that harbored base substitutions from −147 to −145 (Fig. 5A). These mutant constructs were transfected into RAW264.7 cells together with IRF-1 (Fig. 5B). The ISRE mutant (M1) construct lost a significant portion (about two-thirds) of its response to cotransfected IRF-1 (Fig. 5B). Additional mutations...
in the flanks of the ISRE (M2 and M3) did not significantly exacerbate the IRF-1 response (Fig. 5C). These results strongly indicate that the TTTTC motif at −147 to −143 (ISRE) is a major IFN-γ and IRF-1-response element (IRF1-RE) within this promoter region. Interestingly LPS-induced RANTES promoter activity was also partially impaired in these ISRE mutants (Fig. 5D). However, the response to activation by Nf-κB components p50, p65, and c-Rel in the IRF1-RE mutant (M1) was intact (Fig. 5E), suggesting that LPS-induced factors other than Nf-κB may act through this site to induce RANTES gene transcription.

**IRF-1 Binds to the RANTES Promoter Both in Vitro and in Vivo**—To determine whether the transcriptional activation of the RANTES promoter by IRF-1 was a direct event or via an indirect mechanism, we performed EMSAs using nuclear extracts isolated from primary peritoneal macrophages stimulated with IFN-γ or LPS. As shown in Fig. 6A, there was one major IFN-γ-induced nuclear DNA-binding complex formed with an oligonucleotide containing the mRANTES promoter ISRE-IRF1-RE (lane 3) in WT macrophages. This complex was slightly induced by LPS (Fig. 6A, lane 4). In nuclear extracts isolated from IRF-1−/− macrophages, however, the inducible complex was virtually absent under any conditions (Fig. 6A, lanes 5–7). A "supershift" experiment (Fig. 6B) demonstrated that this complex indeed contains predominantly IRF-1 because an anti-IRF-1 antibody was able to retard its mobility (lane 3, indicated by asterisk), and antibodies directed toward several other members of the IRF family, namely IRF-2, IRF-3, IRF-7, and IRF-8 (interferon consensus sequence-binding protein) were not. The IFN-γ-induced IRF-1 binding was abolished when the critical TTTTC IRF1-RE was substituted with GGGG (Fig. 6C), confirming the sequence specificity of the binding. To further confirm the ability of IRF-1 to recognize the ISRE in the RANTES promoter, we expressed IRF-1 via adenovirus-mediated gene delivery in HEK293 cells, which do not express endogenous IRF-1 and thus are free of the concern of "background" issues. As shown in Fig. 6D, a specific complex formed in cells expressing adenovirus-derived IRF-1 (lane 3) but not in cells expressing adenovirus-derived LacZ (lane 2). Moreover, the anti-IRF-1 antibody specifically "shifted" the IRF-1 complex (Fig. 6D, lane 5), but the control antibody did not (lane 4). The top band in Fig. 6D (indicated by a question mark) is likely to contain ISGF3 induced by adenovirus because it has been shown that type-5 adenovirus-transformed mouse fibroblasts have constitutive IFN-stimulated gene factor 3 binding at the ISRE of major histocompatibility complex class I promoter through the production of IFNβ (25). These results demonstrate that IRF-1 specifically and directly interacts with the IRF1-RE at −147 to −143 in the RANTES promoter in vitro.

To determine whether the interaction between IRF-1 and the RANTES promoter occurs in vivo, a chromatin immunoprecipitation assay was performed in mouse peritoneal macrophages. Fig. 6E illustrates the region of the mouse RANTES promoter with the IRF1-RE that was examined in the ChiP assay. Fig. 6F demonstrates that both LPS and IFN-γ induced direct binding of IRF-1 to this region of the RANTES promoter, detected specifically by the anti-IRF-1 antibody (lanes 3 and 4, respectively) but not by the control IgG (lane 4).

**DISCUSSION**

Appropriate recruitment, activation, and regulation of leukocytes are important for eliminating infectious pathogens while at the same time avoiding immune-mediated tissue destruction. Chemokines and their receptors play a central role in this process through their activation and mobilization of leukocytes to sites of infection (26). The localized and controlled chemokine expression profile contributes to the shaping of the immune response. Macrophages represent a leukocyte population involved in the first line of defense.
Regulation of RANTES Transcription by IRF-1

FIG. 4. IRF-1 induces RANTES protein production and promoter activity. A, adenovirus-mediated expression of mouse IRF-1 in HEK293 cells. HEK293 cells were transduced with an adenovirus expression vector containing mouse IRF-1 cDNA (IRF-1/Ad) or LacZ cDNA (LacZ/Ad) for 48 – 72 h. Cytoplasmic (cyto) and nuclear (nu) extracts were isolated and analyzed by Western blot using an anti-IRF-1 antibody. 33 μg of cytoplasmic and nuclear extracts were used in each lane. The positive control was an extract derived from peritoneal macrophages stimulated with IFNγ (4 h). B, WT mouse peritoneal macrophages were transduced with mouse IRF-1 cDNA or LacZ cDNA at different doses of the virus from 10 to 40 plaque-forming units (pfus)/cell for 48 h. RANTES production was measured by ELISA from the supernatants of transduced macrophages (1.0 × 10⁶ cells in 1 ml). Data shown are mean ± S.E. of three independent experiments. C, the full-length mouse RANTES promoter-luciferase reporter construct was transiently transfected into RAW264.7 cells by electroporation. The transfected cells were stimulated with IFNγ, LPS, or IFNγ plus LPS for 7 h and the luciferase activity was measured from cell lysates. Results shown are mean ± S.E. from six independent experiments. D and E, the RANTES promoter-luciferase reporter construct was transiently transfected into RAW cells together with increasing amounts of an IRF-1 expression vector (D) or IRF-2 expression vector (E) or its control vector (pAct-C). Note that the IRF-1, IRF-2, or pAct-C were used in different molar ratios to the reporter, and the total amount of effector in the mixture was maintained at a constant 3.0 μg with varying portions of the effector and the control vector. Luciferase activity was measured from cell lysates. Results shown are mean ± S.E. from three independent experiments. IRF transfected conditions versus medium control with vector alone; *, p < 0.05; **, p < 0.01; ***, p < 0.001. F, kinetic expression of IRF-1 protein. RAW264.7 cells were treated with IFNγ for various times as indicated. 35 μg of nuclear extract were used for Western blot using an anti-IRF-1 or anti-IRF-2 antibody.

against many infections. They have been shown to selectively produce RANTES following infection by herpes simplex virus (HSV) through a mechanism dependent on RNA-dependent protein kinase and infected cell protein (27). RANTES expression in microglia correlates with the clinical onset of experimental autoimmune encephalitis and severity of demyelination (28, 29). The beneficial effects of RANTES to the host in HSV infection was demonstrated by a study in which coinjection with IL-8 and RANTES plasmid DNAs dramatically enhanced antigen-specific Th1-type cellular immune responses and protection from lethal HSV-2 challenge. This enhanced protection appears to be mediated by CD4⁺ T cells and results in reduced HSV-2-derived morbidity as well as reduced mortality (30). The importance of RANTES in virus-induced pathogenesis is also illustrated by studies showing that infection of CD4⁺ T cells by M-tropic strains of HIV-1 is antagonized by the chemokines RANTES, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β, the natural ligands of HIV fusion cofactor CCR5 (31).

Many cell types, including fibroblasts, epithelial cells, and monocytes/macrophages, express RANTES within hours of stimulation by proinflammatory stimuli such as TNFα and IL-1β (32). The human RANTES promoter has been subdivided into five regions (regions A–E) based on deletion studies and reporter gene assays (32–34). There are four binding sites for NFκB proteins that are critical for induction by proinflammatory cytokines TNFα or IL-1β or through the CD28 costimulatory pathway (35). Using fibroblastic or myeloid cells, Génin et al. (36) demonstrated that the kinetics and strength of virus-induced RANTES transcription are highly dependent on the preexistence of IRF-3/7 and NFκB. Direct binding of IRF-3 to the "interferon-stimulated response element" located between −123 and −96 of the human RANTES promoter was also shown with recombinant N-terminal IRF-3 or in 293 cells transfected with a flagged IRF-3 (15).

Several other studies, however, have demonstrated to varying degrees that the RANTES gene is a direct transcriptional target of IRF-1. For example, Miyamoto et al. (37) reported that IL-1β stimulation of RANTES promoter in the human astrocytoma line CH235 involves the direct binding of NFκB and IRF-1. TNFα-induced RANTES gene expression in human colonic subepithelial myofibroblasts requires the direct binding of IRF-1 to the ISRE of the human RANTES promoter (38). The regulatory role and physiological importance of IRF-1 in RANTES expression in...
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vivo was demonstrated in a mouse model of transplant rejection in which Erickson et al. (39) investigated heterotopic heart transplantsations using C57BL/6J (WT) and IRF-1^{-/-} mice as recipients and C3H mice as donors. Median survival time of heart allografts was 8 days in the WT mice and 10 days in the IRF-1^{-/-} mice. Microarray gene expression analysis revealed distinct cytokine and chemokine gene expression profiles in the allografts from the WT and IRF-1^{-/-} recipients. Although expression of IL-4, IL-6, IL-13, MCP-1, MCP-3, and MPIF-2 was up-regulated, RANTES, IL-2Rα and gp130 were down-regulated in allografts from the IRF-1^{-/-} recipients when compared with the WT control, suggesting that IRF-1 controls RANTES expression and RANTES-mediated immune response.

The ISRE-IRF1-RE in mouse RANTES promoter, 5'-ATTTCAGTTTTCTTTTCCCATTTG-3', is identical to the human counterpart except for one nucleotide difference at the position of A (bold-faced and underlined), which is a "G" in the human promoter. The human ISRE has been shown to bind IRF-3 in fibroblast cell lines in response to virus infection (15). By supershift EMSA, however, we could not demonstrate the involvement of IRF-3 in the complex that bound to the mouse RANTES-ISRE in primary macrophages. This difference may be due to species (human versus mouse), or state of the protein (recombinant versus native), or cell types (293 cell line versus primary macrophages). Further investigation is needed to clarify this issue.

Our study demonstrates that IRF-1 is required for RANTES expression by macrophages in response to IFNγ. It represents the first effort to establish a direct causal relationship between IRF-1 and RANTES expression in the context of IFNγ-mediated activation of macrophages by using the IRF-1^{-/-} mice. Our findings show that IRF-1 is dispensable for LPS-induced RANTES expression (Fig. 2, A and E) and that the IRF1-RE at −88 to −79 (17) is not required for NFκB-mediated activation (Fig. 5E). Conversely, the previously described NFκB element at −88 to −79 (17) is not required for IFNγ-IRF-1 response either (data not show). In other words, the ISRE-IRF1-RE and NFκB response element act independently.

In summary, our data have unequivocally established that IRF-1 is an essential and direct transcription mediator for IFNγ-induced RANTES gene expression in macrophages. This is an important pathway because it constitutes a mechanism whereby RANTES expression can be induced in the absence of exogenous pathogens. IRF-1 is also required for maintaining both the basal and MNU-induced RANTES gene expression in vivo. The defining of a novel pathway of RANTES induction via IFNγ and IRF-1 will likely have significant impact on our

**Fig. 5. Mutagenesis of IRF1-RE in RANTES promoter.** A, sequence of the WT mouse RANTES promoter region containing the IRF1-RE (underlined) and that of the ISRE mutants with specific base substitutions (boldfaced and underlined). B–D, the WT RANTES promoter-luciferase reporter construct or the mutant constructs (M1–M3) were transiently transfected into RAW264.7 cells by electroporation, together with the IRF-1 expression vector or its control vector pAct-C (B) or without cotransfection but with cellular stimulation by IFNγ (C) or LPS (D) for 7 h instead. Luciferase activity was measured from cell lysates and expressed as relative activity, i.e. the activity of IRF-1-cotransfected reporter over that of control vector-cotransfected reporter, which was set as 1 (B, D), or the activity of IFNγ- or LPS-stimulated cells over that of unstimulated cells, which was set as 1 (C and D, respectively). Results shown represent mean ± S.E. of three to four independent experiments. E, RAW264.7 cells were transiently transfected with WT or the IRF1-M1 RANTES promoter together with an expression vector for NFκB p50, p65, or c-Rel as indicated. Cells were not further stimulated before luciferase activity measurement. Relative luciferase activity was calculated as fold of NFκB versus its control vector (pMFG) under the medium condition. Data represent the mean ± S.E. from three independent experiments.
understanding of the orchestration of an immune response involving different cell types when macrophages are activated by IFN-γ independently of Toll-like receptors, which recognize pathogens.

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FIG. 6. IRF-1 binds to RANTES promoter both in vitro and in vivo. A, nuclear extracts were isolated from thioglycolate-elicited primary mouse peritoneal macrophages from WT and IRF-1−/− mice following IFNγ or LPS stimulation for 4 h. EMSA was performed with 10 μg of nuclear extract for each sample and a double-stranded oligonucleotide probe containing the −161 to −134 region of the mouse RANTES promoter (sequence given with the critical IRF1-RE underlined). B and D, supershift EMSA was performed with the −161 to −134 probe and nuclear extracts from IFNγ-stimulated macrophages (B) or nuclear extract isolated from HEK293 cells transduced with the adenovirus (Ad) expressing IRF-1 or LacZ (D). A series of IRF antibodies and their control, rabbit IgG, were used. The IRF-1-related complex is indicated by an arrow. Lane 1 of A, C, and D, free probe. The asterisk in B indicates the supershifted IRF-1 complex. The question mark in D indicates a complex of an unidentified nature. E, sequence of mouse RANTES promoter containing IRF1-RE. The sequences of the pair of PCR primers used to perform ChIP are underlined and indicated as the IRF1-RE. F, ChIP analysis was performed in WT mouse peritoneal macrophages. The amplified mouse genomic fragment derived from the endogenous RANTES promoter encompassing the IRF1-RE is indicated. The control antibody was an isotype-matched IgG. Input DNAs were used as controls. Ab, antibody; mut, mutant; mRANTES, mouse RANTES.
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